Adaptation of Recombinant Escherichia Coli LSBJ for PHA Production in a Bioreactor

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Adaptation of recombinant *Escherichia coli* LSBJ for PHA production in a bioreactor

by

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May 2016

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Date: 5/13/16
Abstract

Polyhydroxyalkanoates (PHAs) are plastic biopolymers produced by bacteria under stressful conditions. PHAs are biodegradable and can be made to exhibit similar properties to petroleum-based plastics, which makes mass production of them highly desirable. In this study, we adapted *E. coli* LSBJ for high biomass production in a 2.2 L New Brunswick Scientific BioFlo 310 bioreactor and began studies toward high production of PHA polymer in this setting. With the modified β-oxidation pathway of the LSBJ strain of *E. coli*, the repeating unit length and resulting properties of the PHA polymer can be controlled. The LSBJ strain was modified with several genetic manipulations (Δaas, ΔarcA, Δddl, crp*) to assist in future polymer production in the bioreactor, and a method for high biomass production of the strain in this setting was developed. This method has consistently achieved high cell densities, with optical densities at 600 nm in the range of 30-50 and as high as 74, similar to what has been previously demonstrated for *E. coli* fermentations. This method has also consistently produced biomass several fold higher than shake flask procedures with the LSBJ strain, as evidenced by cell dry weights. With this method fully developed for the 2.2 L bioreactor, PHA production methods at high biomass in this vessel can be investigated beyond the preliminary studies already conducted. Scale-up procedures to larger vessel volumes can then be started, beginning the process toward mass production of PHA using the LSBJ strain.
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Acknowledgements

I would like to thank Dr. Ben Lundgren for his help and instruction in operating the bioreactor, and for overall guidance with other aspects of this project. I would also like to thank Dr. Zaara Sarwar and Ryan Scheel for their help in the operation of the gas chromatography instrument. Lastly I would like to thank Dr. Christopher Nomura for this research opportunity and general guidance during the project.
Introduction

Polyhydroxyalkanoates (PHAs) are plastic biopolymers that are produced in microorganisms as energy storage molecules when exposed to stressful environments. These biopolymers have the potential to replace petroleum-based synthetic plastics, and they also have medical and commercial applications (6). Bacteria such as *E. coli* can be genetically modified with PHA production genes to produce PHAs when fed with fatty acids or simple sugar monomers (5). These systems have the added benefit that, since the PHA production pathway is foreign to them, the bacteria are unable to break down the PHA products, and thus they may instead be harvested.

Traditional methods using shake flasks to grow the bacteria produce limited amounts of PHAs making this method unsuitable for mass production of the polymer (5). The development of a method to mass produce PHA in recombinant *E. coli* to potentially begin the replacement of synthetic plastics with the highly desired, biodegradable PHA plastics was the subject of this study. The short term goal of the study was to develop a method to increase growth and biomass of the LSBJ strain in the bioreactor setting compared to traditionally used shake flasks. The long term goal of this study is to efficiently produce higher quantities of PHA polymer from *E. coli* LSBJ using the bioreactor than shake flask procedures have accomplished.

In order to mass produce PHA using a bacterial system, the culture must be grown to high densities within the bioreactor. A higher density of bacteria per unit volume should theoretically yield a greater amount of polymer produced per unit volume. Previous studies have shown that *E. coli* can be grown to much higher cell densities than typically observed in shake flasks by fed-batch procedures in a bioreactor (7). If PHA-
producing recombinant *E. coli* can be grown to optical densities similar to those achieved by Pfeifer et al. (OD$_{600}$ of 50+), they can then be stimulated and fed the necessary monomers to produce PHA. The high density of the culture should lead to rapid uptake of fatty acids or sugars and their subsequent conversion to PHAs, if the strain is engineered properly. We hypothesize that growing genetically modified *E. coli* to high optical densities via fed-batch procedure prior to induction of the PHA-producing genes will ultimately cause them to produce PHA polymers in higher quantities than seen in traditional shake flask growth experiments. The first step toward this goal was to optimize growth of the polymer-producing strain in the bioreactor, which was accomplished in this study. Preliminary experiments on PHA production were conducted as well.

**Engineering metabolism to provide intermediates for PHA production**

The parent *E. coli* strain used in this study, LSBJ, uses a modified β-oxidation pathway in fatty acid metabolism that grants control of the repeating unit composition of the PHA polymer it produces based on the length of the fatty acid monomers fed to the bacteria (Figure 1) (10). Control of the repeating unit composition of the polymer controls the properties of the produced plastic, which is why this strain was chosen in this study (6).

The LSBJ strain was genetically modified to effectively be able to produce the eventual desired polymer in the bioreactor. Some modifications were made during the increased biomass method development studies, while some modifications were present in the bacteria from the start of the project. Having these genetic changes present during the method development was important to be able to monitor any metabolic and
regulatory changes. It allowed any problems affecting the growth to be resolved prior to the strain being used for PHA production studies. This will prevent complications that could have arisen had these genetic modifications been made after a high biomass production method for the strain had already been developed.

The $dlld$ gene, coding for D-lactate dehydrogenase, was knocked out from the strain because the ultimate desired product to produce in the bioreactor is poly(lactate-co-3-hydroxyoctanoate) [P(LA-co-HO)] copolymer from the bacteria. This copolymer has been shown to have desirable material such as biocompatibility and low toxicity to humans (3). With the $dlld$ gene deleted, the bacteria should be unable to metabolize the D-lactate substrate added to the media and instead shunt it toward copolymer production.

The strain was also engineered with a modified catabolite repressor protein (CRP) gene, $crp^*$. This mutated gene does not eliminate the CRP protein, but instead renders it insensitive to cAMP. This prevents repression of genes in other metabolic pathways, most importantly for this study the $fad$ genes in the $\beta$-oxidation pathway of fatty acid degradation, in the presence of glucose (4). With this mutation, the preferred carbon source for the growth of $E. coli$, glucose, can be used in the bioreactor without repressing the $fad$ genes that are part of the PHA production pathway. Using glucose as the bacteria's carbon source should increase the growth of the culture.

The ArcA protein is a regulatory protein that controls many genes including $fadL$, $fadD$, and $fadE$ in the $\beta$-oxidation pathway. Previous studies in our lab have shown that the deletion of the $arcA$ gene from $E. coli$ LSBJ had led to increased fatty acid uptake and a subsequent increase in PHA production from fatty acids in this strain (9). The $arcA$ gene was deleted from the LSBJ strain used in the bioreactor based on these results.
The *aas* gene encodes the acyl-ACP synthetase enzyme, which is used by the cell to attach exogenous fatty acids to the acyl carrier protein (ACP), which brings them into biosynthetic pathways (8). It has a high affinity for octanoate, which was the fatty acid monomer used in the growth studies. To prevent the use of our fatty acid feed for biosynthesis over PHA production, the *aas* gene was knocked out of the strain to yield the Δ*aas ΔarcA Δddl crp* LSBJ strain that was used in all subsequent fermentations in the bioreactor.

*E. coli* LSBJ has also been shown in previous studies to be able to produce mixed PHAs using other pathways (Figure 2). Wang et al. used the *phaG* gene along with *phaCJ* to produce mixed medium-chain-length PHA polymers from non-fatty acid feedstocks such as glucose (12). Tappel et al. used the *phaA* and *phaB* genes along with the *phaCJ* gene to produce mixed polymers with a majority of PHB polymer (11). These systems were tested in the method development as part of the preliminary polymer production studies in the bioreactor as well.

**Materials and Methods**

**Bacterial strains, plasmids, and media used in this study.** A list of the plasmids used in this study is presented in Table I. The parent strain used was the LSBJ strain with the *crp* mutation and *ddl* gene knocked out already (Δ*ddl crp* LSBJ *E. coli*). This strain was engineered to delete the *arcA* and *aas* genes for later bioreactor fermentations, making Δ*aas ΔarcA Δddl crp* LSBJ the final strain used. Seed cultures were grown on Lennox Broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride) outside of the bioreactor with the antibiotics kanamycin (50 mg/L), ampicillin (100 mg/L), and carbenicillin (100 mg/L) added as needed.
The bioreactor used for this study was the 2.2 L BioFlo 310 bioreactor from New Brunswick Scientific.

In the bioreactor, cells were grown following the fed-batch procedure of Pfeifer et al. (7) with modifications noted below. Feed solutions used included the following:
- glucose feed (55g of (NH₄)₂SO₄, 1.95g of MgSO₄, and 215g of glucose, filter sterilized; appropriate antibiotics steriley added before use), nitrogen-limiting feed (3.3g of (NH₄)₂SO₄, 1.95g of MgSO₄, and 215g of glucose, filter sterilized; appropriate antibiotics steriley added before use), octanoate feed (40 mg/mL of sodium octanoate, filter sterilized), and glycerol feed (55g of (NH₄)₂SO₄, 1.95g of MgSO₄, and 215g of glycerol, filter sterilized; appropriate antibiotics steriley added before use). A solution of K-12 trace minerals (5 g/L of NaCl, 1 g/L of ZnSO₄·7H₂O, 4 g/L of MnCl₂·4H₂O, 4.75 g/L of anhydrous FeCl₃, 0.4 g/L of CuSO₄·5H₂O, 0.575 g/L of boric acid, 0.5 g/L of NaMoO₄·2H₂O, and 4 mL of concentrated sulfuric acid) was used in the initial media of the bioreactor.

**Gene knockout procedure.** Δ*l*ld crp* LSBJ E. coli had been previously created in the lab and was used for the *arcA* and *aas* gene knockouts. The procedure developed by Datsenko and Wanner 2000 was utilized to knock out the genes using the λ red recombinase system (2). Knockout cassettes were generated by PCR using primers of the kanamycin resistance gene encoded in the pKD13 plasmid that were modified with ends homologous to the *arcA* and *aas* genes (Table 2). Δ*l*ld crp* LSBJ E. coli were transformed with the pKD46 plasmid and grown to an OD₆₀₀ of 0.6-1.0. Arabinose was added to a concentration of 0.3% (w/v) to induce expression of the λ red recombinase system. The cells were made electrocompetent and transformed with the knockout
cassettes using a Harvard Apparatus BTX ECM 399 electroporation system. Successful transformants were selected by growing the cells on LB\textsubscript{kan} plates. The pKD46 plasmid was cured from successful transformants by growing at 42°C. Kanamycin resistance was removed from the cassette using FLP recombinase encoded on the pCP20 plasmid, which was subsequently cured by growing successful transformants at 42°C (1, 2). Successful knockouts were verified by PCR using \textit{arcA} and \textit{aas} check primers (Table 2).

**Bioreactor fermentation setup.** All fermentations were set up in the New Brunswick Scientific 2.2 L BioFlo 310 bioreactor in a similar manner to Pfeifer et al. Modifications to this method are noted below in the final developed method for the LSBJ strain. Modifications unique to each individual fermentation leading up to this final method are noted in Table 3. Prior to inoculation, the bioreactor vessel was sterilized by autoclaving while containing 750 mL of F1 salts solution (1.5 g/L of KH\textsubscript{2}PO\textsubscript{4}, 4.34 g/L of K\textsubscript{3}HPO\textsubscript{4}, and 0.4 g/L of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}) with the pH probe, DO probe, and ports in place. The condenser apparatus and any feed bottles used were autoclaved along with the bioreactor vessel. The pH and DO probes were calibrated after the vessel cooled. 15 mL of 50% glucose solution (10 g/L final concentration), 7.5 mL of 200 g/L yeast extract (2 g/L final concentration), 1 mL of 1M MgSO\textsubscript{2} (1.33 mM final concentration), 750 µL of K-12 trace metals solution, and the appropriate antibiotics were added to the vessel media before inoculating. A 50 mL seed culture was grown in LB with appropriate antibiotics overnight at 30°C with shaking at 200 rpm. The seed culture was centrifuged as two aliquots of 20 mL and resuspended in 1 mL of PBS buffer each, then inoculated into the bioreactor. Growth proceeded with pH maintained at 7.00 using 2 M sulfuric acid and concentrated ammonium hydroxide, and temperature was maintained at 37°C. Aeration
was controlled via agitation and filtered air flow through the sparger (GasFlo) to maintain the dissolved oxygen (DO) above 50% according to the cascade outlined in Table 4. The glucose feed was started once the glucose was exhausted from the initial media; this was indicated by a spike in the DO, corresponding to the culture’s decrease in oxygen demand. This feed was initially pumped in at a rate of 15 mL/hour (see Table 3), regulated by a peristaltic pump. When the culture reached a sufficient optical density (typically OD$_{600}$ of above 1.0), the sulfuric acid input was shut off, leaving the bacteria to control the acid level in the culture and the ammonium hydroxide input to prevent the pH from falling below 7.00. When the culture reached an OD$_{600}$ of around 10.0, the temperature was decreased to 30°C, the DO set point decreased to 30%, the glucose feed rate decreased to 10 mL/hr, the octanoate feed started at the appropriate setting (see Table 3) controlled by another peristaltic pump if producing PHA from fatty acid, and IPTG added to an appropriate final concentration (usually 0.5-1 mM, see Table 3). If nitrogen limitation was desired in the fermentation, the glucose feed was switched out for a nitrogen limiting feed and the ammonium hydroxide was replaced with 5 M sodium hydroxide at this time. Growth carried on under these conditions until about 48 hours after inoculation of the bioreactor. To control the foam level in the vessel, a few drops of 10% antifoam were added by the BioFlo 310 system via one of the pumps when the foam level neared the lid of the vessel. Optical density measurements were taken periodically throughout each fermentation along with 5 mL cell and 6 mL cell-free broth samples taken for later analysis. The final culture was frozen at -20°C and saved for potential polymer extraction, with four 20 mL cell samples and one final 6 mL cell-free broth sample taken prior to freezing to analyze with the other samples.
Sample analysis (gas chromatography, GC). HPLC grade chloroform was used for GC sample preparation and analysis. Harvested samples were frozen at -80ºC and lyophilized. The weights of the dried cells and cell-free (CF) samples were recorded, and the samples were used for GC analysis according to the method outlined by Tappel et al. (10). Dried cell and CF samples (10-15 mg) were subjected to methanolysis by dissolving them in 2 mL sulfuric acid:methanol (15:85) and 2 mL chloroform and heating to 100ºC for 140 minutes. The samples were cooled and 1 mL of Nanopure water was mixed into each sample by vortexing. The aqueous and organic layers were allowed to settle for at least 15 minutes, and the organic layer was filtered through a 0.45 µm PTFE syringe filter. A 2 mL GC vial was set up for each sample containing 500 µL of methyl octanoate (1 g/L) in chloroform, and 500 µL of filtered sample was mixed in. Samples were injected using an autoinjector into a GC 2010 Gas Chromatograph with a flame ionization detector for separation. The data was analyzed by Shimadzu’s GC Solution software.

Soxhlet extractions of polymer. End fermentation cell samples were prepared for Soxhlet extractions by washing the cells with an aqueous ethanol solution (30-50%). The cells were lyophilized as a large volume (500+ mL) of culture that had been centrifuged to a pellet. Lyophilized cells were placed in a cellulose thimble which was placed in a Soxhlet extractor, hooked up to a condenser, and set up with 240 mL of chloroform in a round bottom flask. The extraction was performed by refluxing the chloroform through the apparatus for at least 5 hours. The chloroform, which contained the extracted material after reflux, was cooled and evaporated to about 20 mL on a Rotavap at 30ºC. This volume was then filtered through a PTFE filter and cast in a glass petri dish. The dish was
covered in foil poked with holes using a needle and left for the remaining chloroform to evapora
tate in a fume hood. Dried extraction samples were weighed in the dish.

Results

**Biomass increase method development.** Growth results as optical densities at 600 nm (OD$_{600}$) and average cell dry weights (CDWs) of the final culture for each fermentation carried out during the increased biomass method development are recorded in Table 5. Early fermentations (#1-3) using the Δddl $crp^*$ LSBJ strain were fed with both octanoate and lactate in varying amounts (Table 3). These fermentations produced relatively low optical densities of 8.00-17.40 when compared to what Pfeifer et al. (7) were able to accomplish with *E. coli* using their fed-batch procedure (OD$_{600}$ = 50+). CDWs from these fermentations were only marginally higher than those achieved with the LSBJ strain in shake flasks in previous studies, which is typically 0.8-1.7 g/L (Table 5).

The next two fermentations (#4-5) utilized the Δddl $crp^*$ LSBJ strain with the *arcA* gene knocked out. Higher optical densities of 20.00 and 25.30 were achieved in these fermentations, respectively, as well as CDWs about twice as large as the previous fermentations (Table 5). Less octanoate was fed into these cultures and no lactate was used (Table 3).

The next three fermentations (#6-8) were cultured as if attempting to grow PHA polymer from sources other than octanoate. Fermentation #6-7 utilized glucose, and fermentation #8 attempted to use dodecanoic acid (C12). Fermentation #6 yielded a low optical density of 4.98, and a low average CDW of 1.67 g/L (Table 5). Fermentation #7 repeated #6 without IPTG induction, and yielded an optical density of 11.25 and an average CDW similar to fermentations #1-3 and about twice as high as fermentation #6 at
3.52 g/L (Table 5). Fermentation #8 used Brij in the bioreactor media to solubilize the dodecanoic acid. This caused excessive foaming of the culture, which caused it to spill out of the bioreactor and prevented the fermentation from being carried out to completion. No CDWs were recorded as no samples were harvested (Table 3).

Fermentations #9-10 were the first attempted with the aas gene knocked out of the strain. However, due to problems with the dissolved oxygen control (#9) and excess acid addition due to poor pH control resulting from a clogged pipeline that prevented proper airflow (#10), both of these fermentations were terminated early. No samples were harvested and no CDWs were recorded (Table 3).

The next fermentations (#11-15) were fed octanoate and experienced better control of the DO and pH. All fermentations except #13 achieved high optical densities consistent in the range of about 30-50, similar to what Pfeifer et al. (7) were able to achieve. Fermentation #13 experienced excessive spillover of culture due to excess foaming resulting in unquantifiable losses in cell density, but still achieved an optical density of 20.50 and a relatively high CDW of 12.68 g/L. The other fermentations yielded CDWs in the range of 16.69-26.08 g/L (Table 5), which was higher than seen in previous fermentations and at least ten times higher than those typically achieved in shake flasks using the LSBJ strain (10, 12).

Fermentation #16 attempted to use glucose for PHA production as done in fermentations #6-7. It yielded a similar optical density to fermentations #11-15 of 28.50 and a similar cell dry weight as well of 17.13 g/L (Table 5). The optical density was about 2.5 times higher than in fermentation #7, and the CDW was about five times larger.
than achieved in fermentation #7 (Table 5). Fermentation #16 used IPTG induction but at half the concentration of fermentation #6 (Table 3).

Fermentation #17 attempted to use glycerol for PHA production with the pBBRSTQKAB and pTrcGK plasmid system. This fermentation achieved an optical density of 40.80, which was on the higher side of optical densities achieved with the bioreactor, and a cell dry weight of 28.43 g/L, which was higher than any CDW achieved. This fermentation used a glucose feed to grow the culture overnight and switched to a glycerol feed upon induction.

Fermentation #18 attempted to produce PHO from octanoate again. This fermentation was allowed to grow overnight on a glucose feed as was done in fermentation #17, but upon induction the bioreactor was switched to conditions that resembled a shake flask (Table 3). The max optical density achieved was 73.9, higher than any previous fermentation and above the range that Pfeifer et al. achieved. The optical density dropped to 31.7 by the end of the fermentation, and the final CDW of the culture was 18.88 g/L, which was typical of previous fermentations (Table 5).

**Preliminary PHA production.** Gas chromatography (GC) was used to obtain qualitative results from samples taken during the method development as part of preliminary studies on PHA production. All fermentations attempting to produce copolymer (#1-3) or PHO polymer from octanoate (#4-5, #11-15, #18) yielded no polymer by GC analysis. Soxhlet extracted material from fermentations #1, #2 and #11 also showed no polymer was produced by these cultures. No samples were collected from fermentations #8-10 to perform GC analysis on.
Fermentation #6, which was the first growth experiment in the bioreactor that utilized glucose to produce PHAs, did show PHA production by GC analysis. PHO and PHD polymer were produced with increasing amounts toward the end of the fermentation. No PHHx was detected, and a low, unchanging amount of PHDD was detected. Wang et al. (12) used the LSBJ strain to produce PHA polymers from glucose and achieved similar results. They produced high amounts of PHO and PHD, with only minor amounts of PHHx and PHDD. Fermentation #6 produced increasing amounts of exogenous decanoic acid as well according to GC analysis, which was also consistent with results from Wang et al. who believe that this results from over expression of the phaG gene. Fermentations #7 and #16-17, which were also cultured to produce mixed PHA from glucose or glycerol, did not yield any PHA by GC analysis.

**Exogenous fatty acid uptake.** Gas chromatography was also utilized to qualitatively monitor fatty acid uptake from cell and cell-free broth samples in the fermentations that attempted to produce PHO from octanoate (#1-5, #11-15, #18). Fermentations #4-5, which first utilized the arcA deletion strain, showed increased fatty acid uptake compared to fermentations #1-3. Nitrogen limitation and IPTG induction in fermentations #14-15 showed increased fatty acid uptake compared to fermentations #11-13. Fermentation #18 showed a much higher concentration of fatty acid in the cells than in the cell-free broth.

**Discussion**

After several fermentations with manipulations to both the strain and parameters of the growth environment, a consistent, high biomass production method of the LSBJ strain was able to be achieved in the bioreactor. The current method used has consistently achieved optical densities in the range of 30-50 and as high as 74, which is close to the
range of optical densities achieved by Pfeifer et al. (7), whose method this was based on. Cell dry weights (CDWs) have ranged from about 16.7-26.1 g/L, which is about tenfold higher than typical CDWs achieved using shake flasks procedures with the LSBJ strain (10, 12). CDWs in shake flasks with the LSBJ strain typically range from 0.8-1.7 g/L.

This massive increase in biomass production will allow studies toward the long term goal, to produce PHA in higher quantities in the bioreactor than shake flasks achieve with the LSBJ, to be started. The short term goal, which was to develop this method, has been accomplished, but it should be noted that this method can only reliably work using the 2.2 L bioreactor. Development of scale-up procedures to larger bioreactor volumes, such as a 6 L bioreactor, still need to be evaluated using this method.

At the beginning of this method development (fermentations #1-3), CDWs were less than expected and desired. Shake flasks had been previously used to obtain only slightly lower if not equal CDWs to these fermentations (10, 12). Fermentations #1-2 obtained similar optical densities to each other, but #1 had a higher CDW. The reason for this may have been due to the higher feed rate of #2, which fed the bacteria more glucose causing higher lactate and acetate production that may have killed off many of the cells by the end of the fermentation, yielding a lower CDW. Fermentation #3 used much more octanoate compared to #1-2 (5 g vs. 0.8 g). Buildup of excess fatty acid likely prevented this culture from growing as well as previous fermentations. Due to lack of PHA copolymer production in these fermentations, subsequent fermentations attempted to produce only PHO to first see if polymer production could be done in the bioreactor before attempting copolymer production again.
Because of fermentation #3's poor growth, the *arcA* deletion was done in an effort to increase fatty acid uptake to prevent fatty acid buildup from inhibiting the growth of the culture. This deletion was made based on previous studies in the lab (9). More fatty acid was taken up by the bacteria in fermentations #4-5 even though less was fed in, likely because of the *arcA* knockout which was consistent with previous results (9). Higher CDWs and optical densities resulted with these fermentations as well and were also much higher than those typically achieved by shake flasks. Because fermentation #5 exhibited slightly better growth than #4, subsequent fermentations used a lower initial glucose feed rate or switched to a lower glucose feed rate upon induction.

The high biomass production but lack of polymer production by fermentations #4-5 prompted the switch to attempt to utilize glucose to produce PHA using the pBBRSTQK and pTrcGK dual plasmid system. The toxicity of this dual plasmid system when induced inhibited fermentation #6 from growing as well as previous fermentations, but it has been the only fermentation thus far to yield PHA based on gas chromatography analysis. Fermentation #7 did not produce PHA, which shows that IPTG induction is necessary for this system to be expressed. Lower CDWs and optical densities of these fermentations were also due to fact that the temperature was dropped when the optical density was only about 1.0, rather than around 10.0 in previous fermentations. What these fermentations showed was that PHA production from glucose can be achieved by the LSBJ strain in the bioreactor using this system as long as IPTG is used and the bacteria are placed in a stressful environment with PHA-producing conditions.

Fermentations #8-10 were failures in terms of "successful" growth experiments due to early termination and lack of samples and results. What these fermentations
showed was that Brij cannot be used in the bioreactor because the settings (high agitation and GasFlo) will cause excessive bubbling and spilling of the culture and media. They also show that proper control of DO and pH is crucial for and that maintenance of the bioreactor system is important to the optimal growth and control of the culture.

The next series of fermentations (#11-15) saw tremendous increases in biomass production. These fermentations were the first to utilize the \textit{aas} deletion strain with successful high biomass production. The \textit{aas} gene was knocked out because it was believed that the lack of polymer production seen in fermentations #4-5 was because the fatty acid feed was being used preferentially for biosynthesis rather than PHA production due to the extraordinary growth of the culture. Therefore, \textit{aas} was deleted to prevent exogenous octanoate from the feed from being used for biosynthesis so that they could be shunted toward polymer production instead.

Fermentations #11 and #12 used bulk feed strategies to try to force the octanoate into PHO production by overwhelming the pathway with substrate. It was hoped that this strategy would generate PHO production, but this was not the case. The same sort of thinking was used for fermentations #13-15, which instead tried to saturate the pathway by adding excessive amounts of octanoate over a longer time period. This strategy was also used to potentially detect any PHO produced by the bacteria that was beyond the limit of detection of the GC instrument because such small amounts of octanoate (1.5 and 2 g) were being spread across such large amounts of bacteria in fermentations #11-12. This, however, did not result in PHO production either, and analysis of Soxhlet extracted material from fermentation #11 showed that no PHO was produced in this fermentation that would have been beyond the detection limit of the GC instrument.
Fermentations #11-15 consistently achieved CDWs around ten times higher than achieved in shake flasks with the LSBJ strain. The main change made with these fermentations, besides in the fatty acid feed, was stopping the acid feed early in the fermentation. The acid feed was turned off in these fermentations at an optical density of just over 1.0. The constant addition of acid from a 2 M sulfuric acid solution seems to prevent the bacteria from growing to their full potential. When the acid is turned off, pH is controlled by the acids produced and excreted by the bacteria and the base feed (ammonium hydroxide) to keep the pH at around neutral. This allowed the bacteria to grow to notably higher densities during the fermentations.

Fermentation #13 is an anomaly among these five fermentations. It had a lower optical density of 20.50 and a lower CDW of 12.68 g/L than the other four fermentations. The reason for this is because a large amount of octanoate was fed into the culture (8 g), which caused excessive foaming. This caused some of the culture to spill out of the bioreactor, which included an unmeasurable amount of cells. This spillage likely affected the measured optical densities and CDWs for this fermentation, explaining its slightly lower values. This fermentation showed that in the 2.2 L bioreactor, foam levels can be a problem. Headspace above the culture is limited in the smaller bioreactor, which means it is less likely that this will be a problem if the method is scaled up to a larger bioreactor with more headspace.

Fermentation #16 attempted to repeat #6 using the new method for massive biomass production while achieving PHA production. However, no PHA was produced in fermentation #16 despite IPTG induction of the PHA production genes. This is likely because of the tremendous growth of the culture; the conditions were not stressful enough.
for the induction to be enough to get the bacteria to produce polymer. They simply grew too well and never achieved the conditions necessary for PHA production. This fermentation showed that while PHA production from glucose can be achieved in the bioreactor, a more stressful environment will need to be made to stimulate its production.

In an attempt to stress the bacteria more for potential PHA production, fermentation #17 used a glycerol feed after induction. Glycerol is not the preferred carbon source for *E. coli*, and as such should slow the bacterial growth and stress the bacteria. This fermentation also used the PBBRSTQKAB system, which should have yielded high amounts of PHB polymer as an easily observable product. While growing the bacteria overnight on glucose allowed them to achieve a relatively high optical density of about 40, no PHB was observed in the cells by GC. This showed that glycerol was not enough to induce PHA production in the bacteria. They may instead be limited by some nutrients such as coenzyme A, or simply not be starved enough for oxygen to incite PHA production. The CDW in this fermentation, which was 28.43 g/L, is assumed to be a misleading number as the cells never fully dried and were contaminated with glycerol that contributed to the CDW.

To test the limitations of the bacterial culture, fermentation #18 attempted to produce PHO polymer from octanoate using different conditions. The bacteria were allowed to grow overnight on glucose as in fermentation #17, which allowed them to achieve an unprecedented optical density of about 74. This optical density was above the range of optical densities achieved by Pfeifer et al., and shows that the current growth method works very well and is likely optimized. The higher optical density seen in this fermentation compared to fermentation #17 was likely due to the plasmid system used.
The pBBR-C1J4SII plasmid is less toxic than the pBBRSTQKAB and pTrcGK dual plasmid system.

To induce polymer production, this fermentation switched to shake-flask-like conditions. The DO and pH controls were shut off, the agitation was taken off the cascade and lowered to a setpoint of 400 rpm, and the air sparger was turned off along with other regular induction changes (see Table 3). It was believed that by switching to these conditions the bioreactor would simulate a shake flask, completely starving the bacteria for oxygen and nutrients and stressing them via pH to get them to produce polymer.

While the bacteria were clearly stressed, as shown by the decline in optical density to 31.7 by the end of the fermentation, no polymer was produced in this fermentation either. The bacteria must instead not be oxygen limited, or limited by stress, but be limited some other way. Perhaps coenzyme A is limiting, the proteins from the plasmid systems aren’t produced in a high enough amount to compete with other cellular enzymes, or the switch to starvation conditions is too rapid to allow the PHA production enzymes to be produced in the first place.

Octanoate uptake across these fermentations was increased by the \textit{arcA} deletion and nitrogen limitation. These results are consistent with previous studies in the lab (9). Nitrogen limitation helps with fatty acid uptake because octanoate is taken up by AtoC, which is controlled by RpoN, which is regulated by nitrogen.

It is believed that glucose may be repressing the genes of the $\beta$-oxidation pathway despite the \textit{crp*} mutation, but this concept needs to be investigated further. PHA
production from glucose has been shown to be possible in the bioreactor, but this has not been achieved when the newly developed high biomass production method is used.

One potential method to achieve PHO production in the bioreactor using octanoate with this strain is metabolic engineering of the β-oxidation pathway of fatty acids. This will put the entire PHA production pathway from fatty acids under external control, rather than just the PHA production genes, in an effort to force the bacteria to produce polymer when at a high biomass. Current methods of increasing biomass production create an environment (thriving conditions) in the bioreactor that clashes with the conditions necessary for PHA production (starving conditions). Metabolic engineering can force the pathway to be expressed under these conditions.

There are numerous directions that this future study can take. The first step, now that the short term goal has been accomplished, is to work towards the long term goal and generate high PHA production in the 2.2 L bioreactor. High biomass of the LSBJ strain is able to be consistently obtained using the developed method, but it is now a matter of forcing the bacteria to produce PHA polymer at this high density. This may be accomplished through metabolic engineering of the β-oxidation pathway for PHO production from octanoate, and through further manipulation of the bioreactor environment after growing a high density of cells in the vessel such as feeding in pantothenate. Once PHA production is achieved, scale-up procedures to larger vessel volumes can be started.

One area that should also be tested is the effect of antifoam on bacterial growth, PHA production, and PHA quality because antifoam is used, sometimes in large quantities, to control the foam levels of the culture in the bioreactor.
Conclusions

This study had two goals – one short term and one long term. The short term goal, to develop a method for increased biomass production of the LSBJ strain in the bioreactor, has been achieved for the New Brunswick Scientific 2.2 L BioFlo 310 bioreactor system. Optical densities of 30-50 have been consistently achieved from this method with the Δaas ΔarcA Δddl crp* LSBJ strain, and cell dry weights have been attained that are consistently in the range of 16-26 g/L. This increase is around tenfold larger than cell dry weights obtained by shake flask procedures using the LSBJ strain (10, 12). With this goal accomplished, studies toward the long term goal of producing high amounts of PHA polymer in the bioreactor can be started. Preliminary studies show that much needs to be done toward this end, but plans are in place and in time we hope to accomplish this goal as well. This will allow for scale-up procedures to start, beginning the steps toward increased production of PHAs using this modified LSBJ strain.
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   14.

   polyhydroxyalkanoate copolymers from mixtures of plant oils and 3- 


   strategies for improved production of *Escherichia coli*-derived 6- 
   deoxyerythronolide B. Applied and Environmental Microbiology. 68(7): 3287- 
   3292.


Figure 1. Modified β-oxidation pathway of the LSBJ strain using MCL fatty acid substrates for PHA production and lactate for P(LA-co-HA) production. The \textit{fadB} gene and its homolog \textit{fadJ} are knocked out of the strain to prevent removal of carbons from the substrate, allowing control of the repeating unit based on the fatty acid feed length.
Figure 2. PHA polymer production from glucose in the LSBJ strain. The phaA, phaB, phaC1, and PP0763 genes are encoded on the pBBRSTQKAB and pTrcGK plasmids.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD46</td>
<td>λ Red recombinase expression plasmid; expresses $exo$, $\beta$, and $\gamma$ genes from $\lambda$ phage $\text{Par}a\text{B}$ promoter; $araC$; $\text{Amp}^R$; temperature sensitive replicon</td>
<td>2</td>
</tr>
<tr>
<td>pKD13</td>
<td>Neomycin phosphotransferase flanked by FLP recombinase recognition targets, $\text{Amp}^R$, $\text{Km}^R$</td>
<td>2</td>
</tr>
<tr>
<td>pCP20</td>
<td>FLP recombinase expression plasmid; $\text{Amp}^R$, temperature sensitive replicon</td>
<td>2</td>
</tr>
<tr>
<td>pBBR-C1J4SII</td>
<td>pBBR1MCS-2 derivative, $\Delta\text{phaAB, phaJ4, phaC1}$ (STQK)</td>
<td>10</td>
</tr>
<tr>
<td>pBBRSTQK</td>
<td>pBBR1-MCS2 derivative, $Pseudomonas$ sp. 61-3 $\text{phaC1}$ (STQK)</td>
<td>12</td>
</tr>
<tr>
<td>pBBRSTQKAB</td>
<td>pBBR1-MCS2 derivative, $Pseudomonas$ sp. 61-3 $\text{phaC1}$ (STQK), $\text{phaA, phaB}$</td>
<td>11</td>
</tr>
<tr>
<td>pTrcGK</td>
<td>pTrc99A derivative, $P. putida$ KT2440 $\text{phaG}$, PP0736</td>
<td>12</td>
</tr>
<tr>
<td>pBRL 690</td>
<td>pBBR-C1J4SII derivative, $lac$ promoter replaced with $tac$ promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Oligonucleotide primers used for gene knockouts

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD13.F.arcA</td>
<td>ATGCAGACCCCCGACATTTCTTATCGTTGAAAGACGAGTTGGTA ACACGCAAGTGTA6GCTGGAGCTGCTTC</td>
</tr>
<tr>
<td>pKD13.R.arcA</td>
<td>TTAAATCTTCCAGATCACCGCAGAAGCAGATAACCTTACCCTGCTG AGATTTGAAACCTGACGTTGGAGCTGCTTCG</td>
</tr>
<tr>
<td>pKD13.F.aas</td>
<td>ACCACAAACGAAGTGTAGGACGATCGACCTCACTCATCGTGCTG TTGCCAGATCCGACCGCTAGGCTGCTTCG</td>
</tr>
<tr>
<td>pKD13.R.aas</td>
<td>AATCTCCCTCCATTCGCTTTTACTGAATCAGAGCAAGGGAGTTGGAATGTGTAGGCTGAGCTTCAGAGCC</td>
</tr>
<tr>
<td>arcA.check.F</td>
<td>GTAAATTGACAGCATGACAGG</td>
</tr>
<tr>
<td>arcA.check.R</td>
<td>GACGATGAGTACGTATCTGG</td>
</tr>
<tr>
<td>aas.check.F</td>
<td>GCACCTAATTTACTGTCGCTC</td>
</tr>
<tr>
<td>aas.check.R</td>
<td>CACAACGAACTGTTAGTGTGC</td>
</tr>
</tbody>
</table>
Table 3. Fermentation parameter changes for short term goal study fermentations (runs) generating increased biomass of the LSBJ strain in the bioreactor

<table>
<thead>
<tr>
<th>Run</th>
<th>Initial FR (mL/hr)</th>
<th>FR upon induction (mL/hr)</th>
<th>Parameters/changes</th>
<th>IPTG added (mM)</th>
</tr>
</thead>
</table>
| 1\textsuperscript{b} | 12 | 6 | • 11 g lactate, 0.8 g octanoate, bulk feed  
• DO setpoint kept at 50%  
• induction at OD\textsubscript{600} = 15.00 | 0.2 |
| 2\textsuperscript{b} | 15 | N/A | • Repeat of #1 with FR kept high throughout  
• induction at OD\textsubscript{600} = 11.75 | 0.15 |
| 3 | 15 | N/A | • 3 g lactate and 5 g octanoate in glucose feed  
• slow growth; feed rate/temp kept high | None |
| 4 | 15 | N/A | • ArcA knocked out of strain  
• 2 g octanoate fed in separately over 24 hours | None |
| 5 | 7.5 | N/A | • FR kept at half previous rate  
• 2 g octanoate fed separately over 24 hours  
• induction at OD\textsubscript{600} = 14.46 | 1.0 |
| 6\textsuperscript{c} | 10 | N/A | • Temp drop and induction upon feed start  
• induction at OD\textsubscript{600} = 0.9  
• slow growth and early death | 1.0 |
| 7\textsuperscript{c} | 10 | N/A | • Repeat of #6 without IPTG  
• temperature still lowered at feed start | None |
| 8 | 10 | N/A | • Dodecanoate (C12) used as fatty acid  
• Brij added to bioreactor media to solubilize C12  
• culture ended prematurely (excessive bubbles/spillage) | None |
| 9 | 15 | N/A | • \textit{aas} knocked out of strain  
• 0.5g octanoate added in bulk upon  
• induction at OD\textsubscript{600} = 8.14  
• culture spilled over due to excess foam  
• died off <24 hours after inoculation | 1.0 |
| 10 | 15 | N/A | • Changed DO probe membrane  
• 0.5g octanoate added in bulk upon  
• induction at OD\textsubscript{600} = 5.30  
• excess acid added during fermentation due to poor pH control  
• premature death and termination of culture | 1.0 |
11 15 10 • 1.5 g octanoate bulk fed in 0.5 g bunches  • acid feed turned off early starting from here  • culture spilled over due to excess foaming  • None

12 15 10 • 1 g octanoate in glucose feed  • 1 g octanoate fed in bulk as 0.5 g batches  • grew culture for 72 hours  • None

13 15 10 • 8 g octanoate fed in over 24 hours  • excess antifoam added  • culture spilled over due to foaming  • None

14 15 10 • 6 g octanoate fed in over 24 hours  • induction at OD\textsubscript{600} = 8.70  • DO setpoint down to 30%  • nitrogen limited at induction\textsuperscript{e}  • increased antifoam from 5% to 10% concentration prevented culture spillage  • 0.5

15 15 10 • Repeat #14 with new plasmid pBRL 690  • induction at OD\textsubscript{600} = 17.70  • 0.5

16\textsuperscript{c} 15 10 • Attempt to produce PHA from glucose  • induction at OD\textsubscript{600} = 13.24  • 0.5

17 15 10 • Attempt to produce PHA from glycerol  • pBBrSTQKAB system used  • induction at OD\textsubscript{600} = 40.80  • grown on glucose overnight, glycerol upon induction  • 1.0

18 15 10 • grown on glucose overnight, glycerol upon induction  • induction at OD\textsubscript{600} = 73.90  • 6 g octanoate fed in over 24 hours  • upon induction: pH/DO control off, agitation to 400 rpm, air off, cascade off  • 1.0

\textsuperscript{a} FR = feed rate, of the glucose feed  
\textsuperscript{b} Fermentations with the strain that included pZS4II for PLA/copolymer production  
\textsuperscript{c} Fermentations with the strain for PHA production from glucose/glycerol using pBBrSTQK(AB) and pTrcGK, no octanoate added
Table 4. Cascade settings created on the BioFlo 310 bioreactor system to control dissolved oxygen (DO) during fermentations

<table>
<thead>
<tr>
<th>To</th>
<th>Start Setpoint</th>
<th>DO Start Out %</th>
<th>End Setpoint</th>
<th>DO End Out %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation (rpm)</td>
<td>200</td>
<td>0.0</td>
<td>950</td>
<td>50.0</td>
</tr>
<tr>
<td>GasFlo</td>
<td>5.0</td>
<td>50.0</td>
<td>20.0</td>
<td>90.0</td>
</tr>
</tbody>
</table>
Table 5. Maximum optical density at 600 nm and cell dry weights of the short term study fermentations

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Max OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Cell dry weight (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.00</td>
<td>4.51 ± 0.36</td>
</tr>
<tr>
<td>2</td>
<td>17.40</td>
<td>3.03 ± 0.40</td>
</tr>
<tr>
<td>3</td>
<td>8.00</td>
<td>2.88 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td>20.00</td>
<td>7.29 ± 0.13</td>
</tr>
<tr>
<td>5</td>
<td>25.30</td>
<td>8.00 ± 0.23</td>
</tr>
<tr>
<td>6</td>
<td>4.98</td>
<td>1.67 ± 0.12</td>
</tr>
<tr>
<td>7</td>
<td>11.25</td>
<td>3.52 ± 0.08</td>
</tr>
<tr>
<td>8</td>
<td>1.57</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>8.14</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>5.30</td>
<td>N/A</td>
</tr>
<tr>
<td>11</td>
<td>35.00</td>
<td>19.54 ± 0.17</td>
</tr>
<tr>
<td>12</td>
<td>48.20</td>
<td>26.08 ± 0.97</td>
</tr>
<tr>
<td>13</td>
<td>20.50</td>
<td>12.68 ± 0.14</td>
</tr>
<tr>
<td>14</td>
<td>33.75</td>
<td>17.13 ± 0.10</td>
</tr>
<tr>
<td>15</td>
<td>27.70</td>
<td>16.69 ± 0.18</td>
</tr>
<tr>
<td>16</td>
<td>28.50</td>
<td>17.13 ± 0.12</td>
</tr>
<tr>
<td>17</td>
<td>40.80</td>
<td>28.43 ± 0.40</td>
</tr>
<tr>
<td>18</td>
<td>73.90</td>
<td>18.88 ± 0.12</td>
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