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Deregulation of fatty acid transport in *Escherichia coli* for enhanced control of biodegradable plastic copolymer production

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May 2014

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Abstract

Polyhydroxyalkanoates (PHA) are one possible alternative for petroleum-based plastics that have been shown to be produced effectively in bacterial systems utilizing fatty acids. The global regulatory genes *arcA* and *ompR* are known to regulate steps involved in fatty acid transport and metabolism, making them promising candidates for research. In this study, we deleted the *arcA* and *ompR* genes in order to deregulate fatty acid uptake. To measure the effect of these deletions, copolymers were produced using recombinant *E. coli* and the repeating unit composition was analyzed. Residual fatty acid levels in media were also measured to determine a difference in uptake. Fatty acid transport was significantly altered in the *arcA* mutant, which depleted twice as much dodecanoic acid as the control strain LSBJ. The *arcA* mutant also showed significantly increased PHA production, measured at 30.64% (by weight) PHA, compared to 21.47% in LSBJ. Other mutants did not show significant changes. These results show that *arcA* is a promising mutant for increased production of PHA, and that increasing intracellular dodecanoic acid concentration has no effect on the ratio of monomers in PHA produced. This could open up new areas of research into producing plastics with very specific properties, giving PHAs a wide array of modern applications.

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Acknowledgements

I would like to thank Lucia Salamanca-Cardona for her instruction and assistance in the operation of the gas chromatography instrument. I would also like to thank Dr. Ben Lundgren for his advisement in laboratory protocols necessary for the successful completion of this project.

Introduction

The accumulation of enduring petroleum-based plastics is a continuing problem for modern societies, and there is an increasing demand for biodegradable alternatives. Polyhydroxyalkanoates (PHAs) are a class of biodegradable polymers that are produced in many microorganisms as a form of carbon storage, and have been shown to be produced effectively in the lab using bacterial systems[1]. The purpose of this research was to determine if certain gene knockouts could improve the efficiency of fatty acid uptake in *E. coli* LSBJ, a strain produced by this lab during previous work, and potentially increase overall yield[2]. This could in turn make PHA copolymer production a more viable alternative to conventional petroleum-based plastic production by increasing the control and efficiency of this system.

The current system of PHA production in *E. coli* LSBJ utilizes a modified β-oxidation pathway to produce polymers with precisely controlled repeating unit compositions (Figure 1). Fatty acid substrates of specified carbon lengths are incorporated as PHA monomers of an equal carbon length, allowing a very high degree of control[2]. Other studies have shown that this level of control is also present in the production of PHA copolymers, and the ratio of fatty acid substrates can be manipulated to produce desired monomer ratios[3].

We identified two global regulatory genes, *arcA* and *ompR*, which both regulate *fad* genes involved in fatty acid uptake and metabolism. The *arcA* gene regulates several aerobic metabolic pathways when the bacteria is exposed to anaerobic conditions,

including several genes involved in fatty acid uptake and metabolism[4]. These regulated genes include *fadL*, *fadD*, and *fadE,* which all play an important role in the modified β-oxidation pathway used by *E. coli* LSBJ[2, 4]. The *ompR* gene regulates *fadL* specifically, which codes for long-chain fatty acid transport proteins, and represses the transcription of *fadL* under conditions of high osmotic concentration[5, 6]. A diagram illustrating the regulation of both genes is shown in Figure 2. Our rationale for this experiment was that the deletion of these two genes, both individually and together in one strain, would deregulate expression of the *fad* genes and subsequently increase the expression of proteins involved in our PHA production pathway.

Materials and Methods

Materials: A complete list of strains and plasmids used in this study is shown in Table 1. All strains were grown on Lennox Broth (LB; composition per liter: 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride) purchased from Difco, and the antibiotics kanamycin (50 mg L^{-1}) and ampicillin (100 mg L^{-1}) were added to media throughout the experiment as appropriate. The fatty acids decanoic acid (Alfa Aesar) and dodecanoic acid (Acros Ogranics) were used as substrates for PHA production, along with the surfactant Brij-35 (Fisher Scientific) at a concentration of 4.0 g L^{-1}. ACS reagent-grade chloroform was used for gas chromatography sample preparation.

Gene knockouts: A protocol developed by Datsenko and Wanner[7] for the inactivation of chromosomal genes in *E. coli* was followed for the removal of the *arcA* and *ompR* genes from *E. coli* LSBJ in two distinct strains, as well as simultaneously in a single strain.

Primers for the kanamycin gene in pKD13 were designed with ends homologous to *arcA* and *ompR* (Table 2). The knockout cassettes were generated using Primestar HS DNA polymerase (Takara) and an iCycler thermal cycler (Bio-rad) as per manufacturer recommendations. *E. coli* LSBJ was transformed with pKD46 and the expression of the λ red system was induced by the addition of L-arabinose to a concentration of 0.3% (w/v). These cells were grown to an OD_{600} of 0.6 to 1.0, made electrocompetent, and transformed with the PCR-generated knockout cassettes using an ECM 399 electroporator (BTX) at 1500 V referencing the manufacturer's protocol for *E. coli*. Successful transformants were selected on LB-agar plates containing kanamycin, and the temperature sensitive plasmid pKD46 was removed by growth at 42°C. The antibiotic cassettes were removed by the expression of FLP recombinase from the pCP20 plasmid, and this temperature-sensitive plasmid removed from cells by growth at 42°C. Each deletion was confirmed by PCR using loci check primers (Table 2).

PHA production in all strains: Protocol for PHA production and cell harvest was adapted from previous publications[2], and any modifications to this protocol are noted. *E. coli* LSBJ, RSC02, RSC04, and RSC06 were made chemically competent and transformed with pBBR-C1J4SII, following standard procedures[8], to express PHA synthase and enoyl-CoA hydratase. Transformants were grown on LB-agar plates with kanamycin, and single colonies were used to inoculate separate 2 mL LB cultures with kanamycin, in triplicate for each strain, an alteration to the previous protocol. Cultures were grown for 12-16 h at 37°C and 200 rpm. One milliliter of each culture was used to inoculate 100 mL of growth media in 500-mL baffled shake flasks. Growth media contained LB, kanamycin,

Brij-35, and both decanoic and dodecanoic acid at a mole ratio of 70:30 to a final concentration of 2.0 g L^{-1}. The cell cultures were grown for 48 hours at 30°C and 250 rpm on a rotary shaker. After the growth period, cells were harvested using previously published methods[2], with one alteration; 5 mL of supernatant were collected after the first centrifugation and filter-sterilized (0.45 µm PES, VWR) into pre-weighed 15-mL centrifugation tubes (brand). The fully washed and harvested cells were resuspended in 3 mL of Nanopure filtered water (Barnstead), both the cells and the filtered supernatant frozen at -80°C, and dried via lyophilization.

Gas chromatography (GC) analysis: The repeating unit composition of PHAs were determined using GC, as previously described[2]. Dried cells and supernatant (15-20 mg) were dissolved in 2 mL of sulfuric acid:methanol (15:85) and 2 mL of chloroform and heated at 100°C for 140 min. The samples were cooled to room temperature, 1 mL of Nanopure filtered water added, and mixed by vortexing. Aqueous and organic layers were separated by centrifugation at 700 rpm for 2 min. The organic layer was passed through a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter (Restek). A sample of 500 µL of each filtered sample was mixed with 500 µL of caprylic acid (1 g L^{-1}) in chloroform in a 2 mL GC vial. Samples were injected and separated in a GC 2010 Gas Chromatograph with an AOC-20i autoinjector and a flame ionization detector as previously described[2]. Shimadzu's GCSolution software was used to analyze the data.

Results

Residual fatty acid analysis: Analysis of dried supernatant by GC showed the composition of free fatty acids not taken up by the cells, for each strain, as percentage of total weight (Figures 3, 4). Residual dodecanoic acid was lowest in *E. coli* RSC02, and was twice as depleted as *E. coli* LSBJ, while the other RSC strains had significantly elevated levels of residual dodecanoic acid (Figure 3). Residual decanoic acid was lowest in *E. coli* LSBJ, though not significantly so compared to *E. coli* RSC02, and the other two RSC strains again had significantly elevated levels of residual decanoic acid (Figure 4).

PHA accumulation in all strains: Analysis of dried cells by GC for PHA composition and abundance revealed that *E. coli* RSC02 produced significantly more PHA than *E. coli* LSBJ, while the other two RSC strains did not produce significantly different amounts compared to each other or to LSBJ (Figure 5). The cell dry weight (CDW) of RSC02 was also significantly higher than the other strains, and differences were seen between all strains (Table 3). None of the strains showed a significant difference in the repeating unit composition of the PHA polymers (Table 3).

Discussion

From the results it can be seen that *E. coli* RSC02 has improved performance over the parent strain, *E. coli* LSBJ. These results show that PHA production varied across strains with RSC02 producing significantly more polymer than LSBJ at 30.64% by weight (Figure 5). There was no statistically significant difference between the other strains. The RSC02 strain also depleted twice as much DDA from the media, while using slightly

less DA than LSBJ (Figures 3, 4). Despite this vast difference in fatty acid uptake, the composition of repeating units was not changed significantly. This shows that fatty acid uptake is not a significant determining factor in PHA composition in our current system, as increased uptake of DDA had no effect on the ratio of 3-hydroxydodecanoate (3HDD) to 3-hydroxydecanoate (3HD). This also shows that some fatty acids are taken up preferentially. It is possible that enzyme specificity has an effect on this preferential uptake, and a greater effect on copolymer repeating unit composition than intracellular substrate availability.

The other strains produced in this study, RSC04 and RSC06, did not perform as well as the parent strain LSBJ. Since RSC06 is a double knockout strain, it is likely that the *ompR* deletion in both of these strains is the cause of this poor performance. This could be attributed to a detrimental effect to the health of bacterial cells, as the cell dry weights of strains containing this mutation were significantly lower (Table 3). The regulatory effects of *ompR* are complex and not fully characterized, and additional regulation by this gene may be important for healthy cellular function[5].

Further testing is needed to strengthen these findings, particularly as there were some strong outliers, and research is currently underway. These preliminary results provide a foundation for additional research into the effects of the *arcA* mutation on the transport and incorporation of other fatty acid substrates. In addition, other analytical methods should be used to verify these results and to determine if the properties of produced copolymers remain unaltered.

Conclusion

This research sought to determine the effects of two global regulatory genes, *arcA* and *ompR,* on the production of PHA copolymers in *E. coli* LSBJ. These two genes were deleted to deregulate genes involved in fatty acid uptake and metabolism. The deletion of the *arcA* gene successfully increased the uptake of dodecanoic acid, one of the two fatty acid substrates present, and also increased the total PHA produced. The deletion of *arcA* had no effect on the ratio of repeating units in the copolymer produced.

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Tables and Figures

Table 1: Strains and Plasmids

Strain/Plasmid	Relevant characteristics	Source or reference
Strains		
E. coli LSBJ	*E. coli* LS5218 *fadB::Cm,* Δ*fadJ*	2
E. coli RSC02	*E. coli* LSBJ Δ*arcA*	This study
E. coli RSC04	*E. coli* LSBJ Δ*ompR*	This study
E. coli RSC06	*E. coli* RSC04 Δ*arcA*	This study
Plasmids		
pKD46	Λ Red recombinase expression plasmid; expresses *exo, β,* and *γ* genes from λ phage; P_{araB} promoter; *araC*; Amp^R; temperature sensitive replicon	6
pKD13	Neomycin phosphotransferase flanked by FLP recombinase recognition targets, Amp^R, Km^R	6
pCP20	FLP recombinase expression plasmid, Amp^R, temperature sensitive replicon	6
pBBR-C1J4SII	pBBR1MCS-2 derivative Δ*phaAB*, *phaJ4, phaC1* (STQK)	2

All strains and plasmids used during this study.

Table 2: Oligonucleotide Primers

Primers	Sequence* (5’ to 3’)
pKD13.F.*arcA*	ATGCAGACCCCGCACATTCTTATCGTTGAAGACGAGTTGGTAACACGCA AGTGTAGGCTGGAGCTGCTTC
pKD13.R.*arcA*	TTAATCTTCCAGATCACCGCAGAAGCGATAACCTTCACCGTGAATGGTG GATTCCGTGGATCCGTCGACC
pKD13.F.*ompR*	ATGCAAGAGAACTACAAGATTCTGGTGGTCGATGACGACATGCGCCTG CGGTGTAGGCTGGAGCTGCTTC
pKD13.R.*ompR*	TTAGAACATTACCTTATGACCGTACTGCTCAAGAATGCCTTTCACGCGTT ATTCCGTGGATCCGTCGACC
arcA.check.F	GTTAATTTGCAGCATGCATCAGG
arcA.check.R	GACGATGAGTTACGTATCTGG
ompR.check.F	AAATTGTTGCGAACCTTTGG
ompR.check.R	GCAATAACGTACGGGCAAAT

*Underlined sequences are areas homologous to the gene being knocked out.

Table 3: PHA Yields and Composition

			PHA composition (mol%)	
Strain	CDW (g/L)	PHA (wt %)	3HD	3HDD
LSBJ	1.33 ± 0.06	21.47 ± 2.64	40.07 ± 2.36*	59.93 ± 2.36*
RSC02	1.50 ± 0.03	30.64 ± 1.47*	39.89 ± 0.13	60.11 ± 0.13
RSC04	0.83 ± 0.02	23.12 ± 3.70	39.00 ± 1.86	61.00 ± 1.86
RSC06	0.97 ± 0.02	25.58 ± 4.58	38.09 ± 0.72	61.91 ± 0.72

Cell dry weight (CDW), PHA production, and composition in each knockout strain compared to parent LSBJ strain. All values are averages of biological triplicates, ± the standard deviation. Average composition in LSBJ would have been more similar to other strains, but was affected by an outlier.**Calculated as duplicates due to strong outliers*

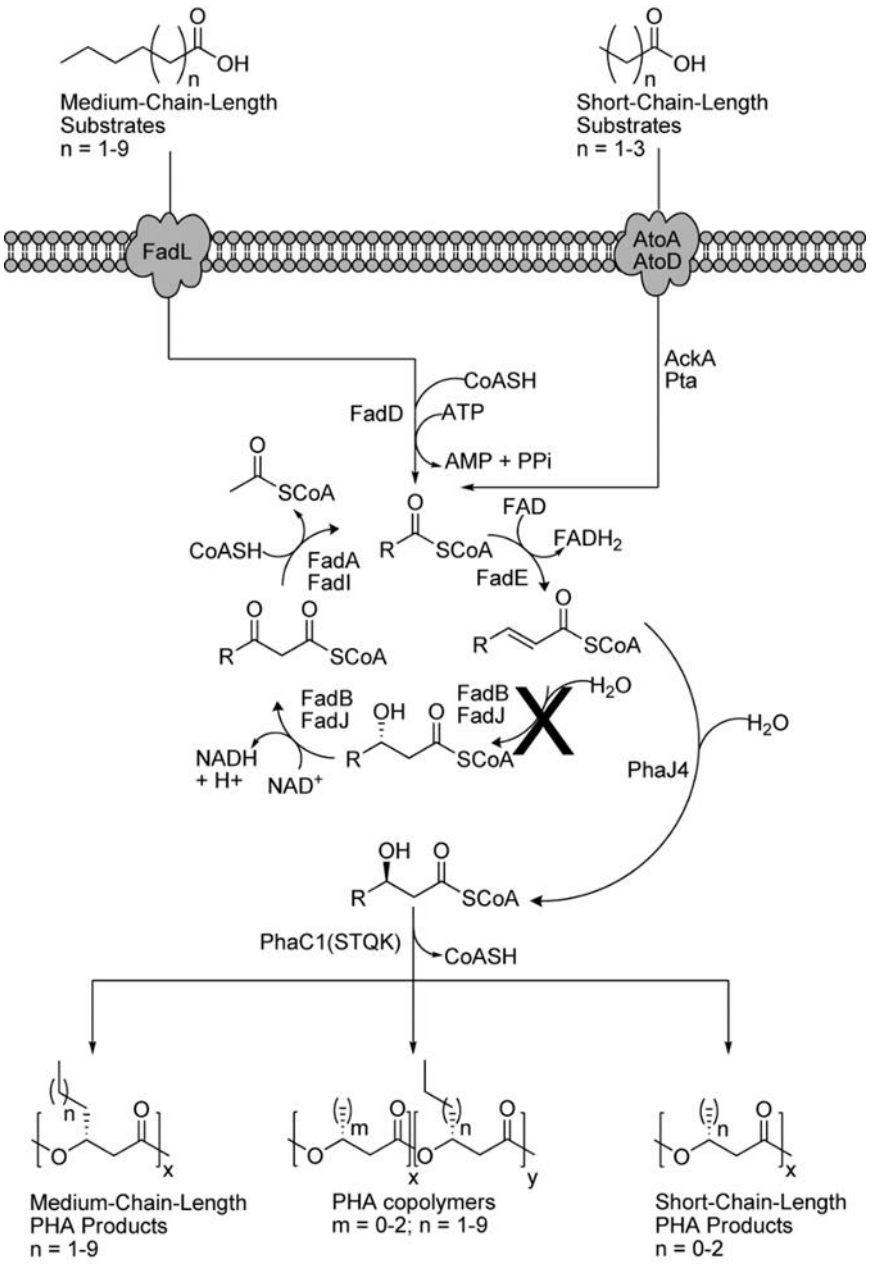

Figure 1: The modified β-oxidation pathway of *E. coli* LSBJ. This was the parent strain for each knockout in this study[2]. Expression of pBBR-C1J4SII provides the PhaJ4 and PhaC1 (STQK), which correspond to enoyl-CoA hydratase and PHA synthase respectively.

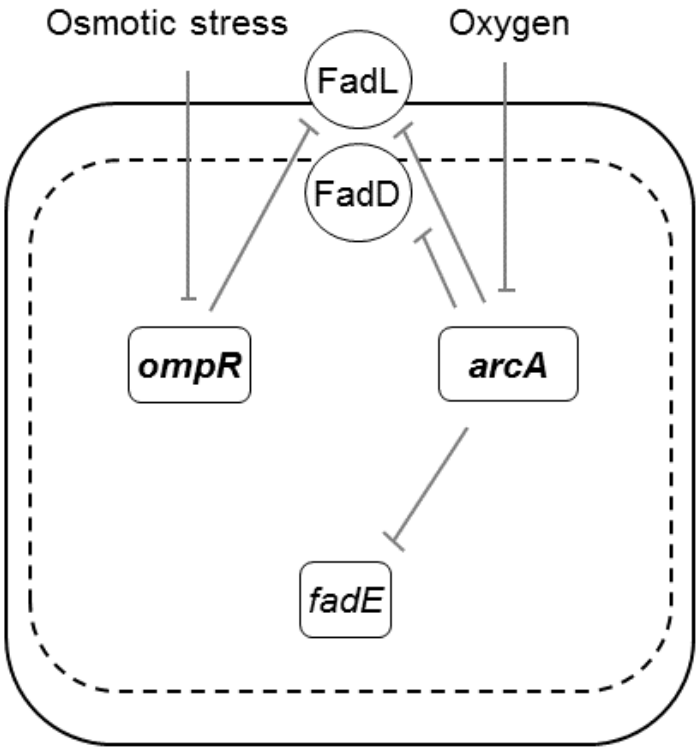

Figure 2: Regulation effects of *ompR* and *arcA* on the beta-oxidation pathway; blunt-ended arrows imply inhibition.

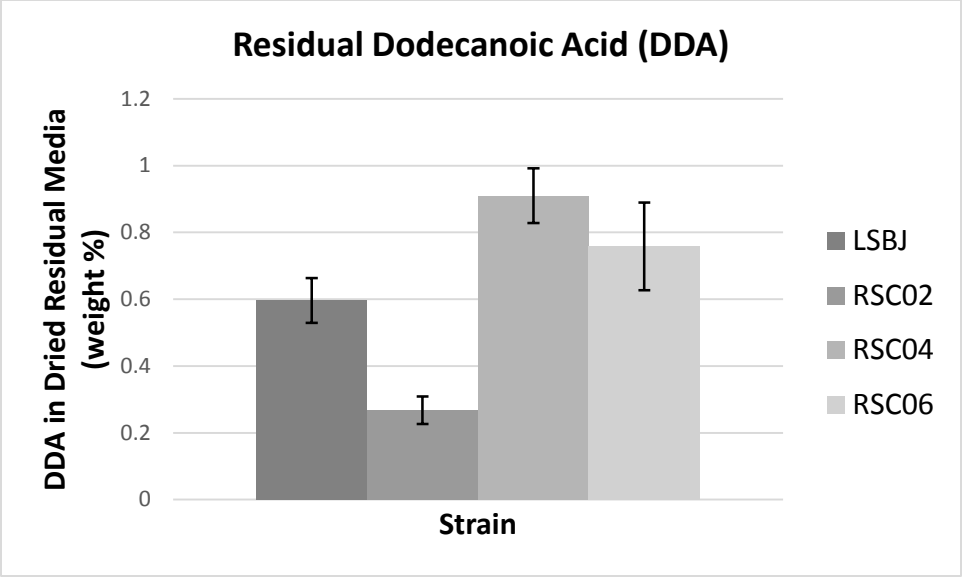

Figure 3**:** Residual DDA in spent media, used as a measure of comparison of fatty acid uptake. The RSC02 depleted the DDA twice as much as LSBJ, while RSC04 and the double knockout took up significantly less. All values are averages of triplicate experiments, ± the standard deviation.

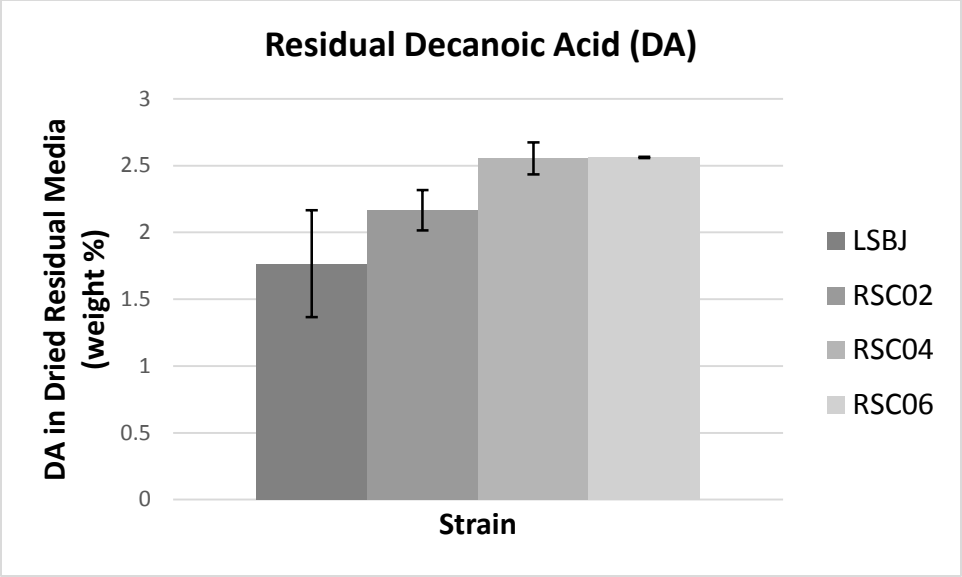

Figure 4: Residual DA in spent media, used as a measure of comparison of fatty acid uptake. There is a slightly decreased uptake of DA by RSC02 compared to LSBJ, and the other strains have significantly less than RSC02. All values are averages of triplicate experiments, ± the standard deviation.

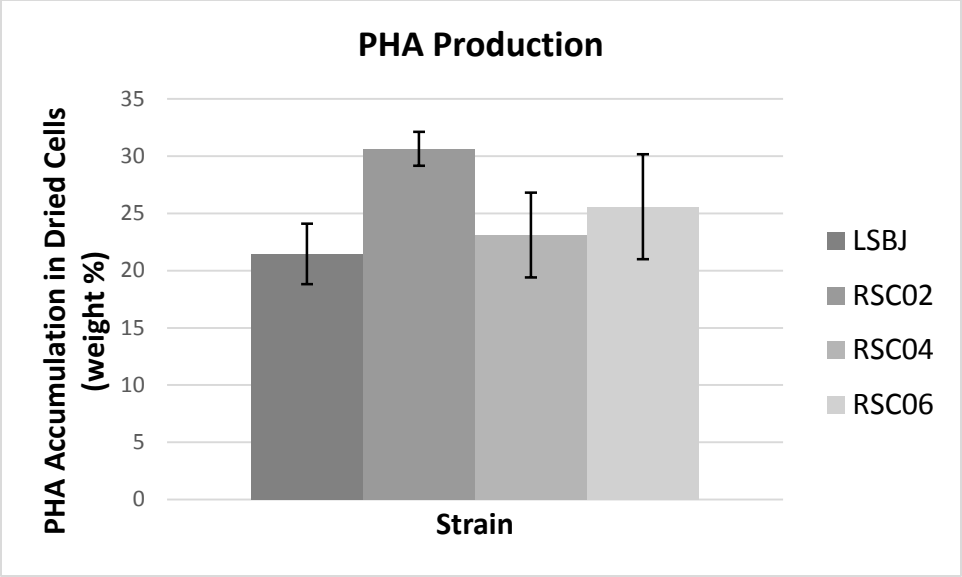

Figure 5: PHA produced by each strain as a percent of cell dried weight. The RSC02 has the highest production at 30.64% with a standard deviation of 1.47%. All values are averages of triplicate experiments, ± the standard deviation, with the exception of RSC02 (calculated as a duplicate due to a strong outlier).