A Forward-Design Approach to Increase the Production of Poly-3-Hydroxybutyrate in Genetically Engineered *Escherichia coli*

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Abstract

Biopolymers, such as poly-3-hydroxybutyrate (P(3HB)) are produced as a carbon store in an array of organisms and exhibit characteristics which are similar to oil-derived plastics, yet have the added advantages of biodegradability and biocompatibility. Despite these advantages, P(3HB) production is currently more expensive than the production of oil-derived plastics, and therefore, more efficient P(3HB) production processes would be desirable. In this study, we describe the model-guided design and experimental validation of several engineered P(3HB) producing operons. In particular, we describe the characterization of a hybrid *phaCAB* operon that consists of a dual promoter (native and J23104) and RBS (native and B0034) design. P(3HB) production at 24 h was around six-fold higher in hybrid *phaCAB* engineered *Escherichia coli* in comparison to *E. coli* engineered with the native *phaCAB* operon from *Ralstonia eutropha* H16. Additionally, we describe the utilization of non-recyclable waste as a low-cost carbon source for the production of P(3HB).

Introduction

Conventional oil-derived polyolefin plastics exhibit useful characteristics that have extensive commercial applications. However, the accumulation of plastics in the environment and the non-renewable source of polyolefin plastics have stimulated interest in sustainable sources of plastic production. Biopolymers, such as poly-3-hydroxybutyrate (P(3HB)), are produced as a carbon store in an array of organisms and exhibit characteristics which are similar to oil-derived plastics [1,2]. Furthermore, P(3HB) has the added advantages of biodegradability, biocompatibility and, based upon several life cycle analyses, P(3HB) production is more environmentally sustainable than polyolefin plastic production [1,3,4]. Despite these advantages,
P(3HB) production is currently more expensive than the production of oil-derived plastics, and therefore, more efficient P(3HB) production processes would be desirable [5].

Genetic engineering approaches—in which the P(3HB)-producing operon, phaCAB, is cloned into *Escherichia coli*—have pioneered the industrial production of P(3HB) [6]. More recently, synthetic biology approaches involving the rational engineering of the phaCAB operon [7], and metabolic engineering strategies [8] have continued to increase the efficiency of P(3HB) production. In this paper we report on the forward-design and experimental validation of a rationally engineered phaCAB operon with a hybrid promoter design. Additionally, we describe the utilization of non-recyclable waste as a low-cost carbon source for the production of P(3HB).

### Results and Discussion

The phaCAB operon from *Ralstonia eutropha* H16, is the most extensively studied P(3HB) synthesis operon [2,9]. It consists of three enzymes, which through a multi-stage enzymatic process generate P(3HB) inside the cell from the central metabolite acetyl-CoA [1]. The process is shown in Fig. 1A and is briefly summarised here. Firstly, PhaA (3-ketothiolase) combines two molecules of acetyl-CoA to form acetoacetyl-CoA. Next, PhaB (acetoacetyl-CoA reductase) reduces acetoacetyl-CoA to form (R)-3-hydroxybutyl-CoA, which is then polymerised by PhaC (PHA synthase) to form poly-3-hydroxybutyrate P(3HB). The phaCAB operon from *R. eutropha* H16 was originally cloned into *E. coli* in the late 1980s [2]. More recently the Tokyo Tech 2012 iGEM team (http://2012.igem.org/Team:Tokyo_Tech) cloned, characterised and standardized the native phaCAB operon (BBa_K934001) into a biobrick-compatible format for the synthetic biology community.

In order to increase P(3HB) production, this study used a forward-design approach that is based upon the engineering principles of synthetic biology [10]. Several phaCAB operons were rationally engineered (Fig. 1B). The constitutive phaCAB operon (BBa_K1149052) was...
designed such that the native promoter and RBS were replaced with the constitutive promoter
J32104 from the Anderson collection [11] and the RBS B0034. The hybrid
phaCAB operon de-
sign (BBa_K1149051) was constructed in parallel to the constitutive operon and was noted for
its dual promoter and RBS combinations (Fig. 1B). Design considerations were based upon
modeling simulations of the PhaCAB pathway in engineered E. coli. The model was comprised
of the glycolysis pathway, the tricarboxylic acid (TCA) cycle and the
phaCAB synthetic path-
way (S1 Supporting Information; S2 Supporting Information). These pathways were coupled
in order to reflect the metabolic flux [12] of several metabolites and species between them and
to provide a qualitative indication of their influence on P(3HB) production.

A sensitivity analysis of species within the synthetic pathway revealed that increasing ex-
pression of phaB would be critical for directing the reaction flow towards the end product—an
increase in P(3HB) production (S1 Supporting Information; S2 Supporting Information). In
order to increase expression of phaB, further simulations predicted that of the several simulated
phaCAB operon designs, an operon that incorporates the constitutive promoter, J23104, would
result in the greatest increase in P(3HB) production (Fig. 2).

To experimentally validate our forward-design approach, E. coli MG1655 carrying either the
empty vector (BBa_K608002), native phaCAB (BBa_K934001), constitutive phaCAB
(BBa_K1149052) or hybrid phaCAB (BBa_K1149051) operons were cultured with Lysogeny
broth (LB) media, supplemented with 3% glucose (w/v) for either 24 or 48 hours. P(3HB) was
purified from each of the engineered populations using sodium hypochlorite and were weighed
for comparison (Fig. 3A). Average P(3HB) production at 24 hours was around two-fold higher
in constitutive phaCAB-engineered E. coli (0.49 g/L S.D. ± 0.06) compared to native phaCAB
(0.22 g/L S.D. ± 0.18), while average P(3HB) production was six-fold higher in hybrid phaCAB-
engineered E. coli (1.47 g/L S.D. ± 0.48). At 48 hours, P(3HB) production was around three-fold
higher in constitutive phaCAB-engineered E. coli (1.05 g/L S.D. ± 0.26) and hybrid phaCAB-
engineered E. coli (0.94 g/L S.D. ± 0.14), compared to native phaCAB (0.30 g/L S.D. ± 0.14)
(Fig. 3A). In hybrid phaCAB-engineered E. coli we observed a decrease in P(3HB) content, ex-
pressed as a percentage of the cell dry weight (CDW) from 50% at 24 hours to 32% at 48 hours.

Fig 2. Simulated P(3HB) production in phaCAB-engineered E. coli. In order to simulate P(3HB)
production in phaCAB-engineered E. coli, a P(3HB) synthesis model was constructed using the Simbiology
toolbox of Matlab. Using this model the flux of several metabolites and species were simulated in order to
identify aspects of the system that could be selectively tuned to increase the production of P(3HB). From
these analyses, several novel phaCAB operons were designed. These data show the simulated P(3HB)
production across several different phaCAB operon designs, where phaCAB expression is under the control
of the indicated Anderson constitutive promoters.
Fig 3. P(3HB) production in phaCAB-engineered E. coli. E. coli MG1655 transformed with empty vector, native, constitutive or hybrid phaCAB constructs were cultured in 1 liter LB media, supplemented with 3% glucose (w/v) for 24 hours or 48 hours. P(3HB) was purified from these cultures and measured as (A) P(3HB) production (g/L) and (B) P(3HB) content (weight [wt.] % of cell dry weight [CDW]). Data represent the mean +/- the standard deviation of three independent experiments. Student t-test, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

doi:10.1371/journal.pone.0117202.g003
Similar observations have been previously reported, and is understood as a consequence of competition for cellular metabolites between cell growth and P(3HB) production [13–15]. The empty vector-engineered E. coli did not produce detectable levels of P(3HB).

Qualitative analysis of Nile Red stained E. coli also confirmed that the constitutive and hybrid phaCAB operon designs increased P(3HB) production (S3 Supporting Information). It is likely that the dual promoter and RBS design (Fig. 1B) of the hybrid system results in a higher level of mRNA transcript production and/or ribosome recruitment and thus an increase in the expression of the PhaCAB enzymes. qRT-PCR analysis, confirmed that both the hybrid and constitutive phaCAB operon designs result in higher levels of phaCAB transcripts than that of the native phaCAB operon (S6 Supporting Information).

Interestingly, as shown in Fig. 3A, P(3HB) production from the hybrid operon at 24 h was greater than the combined P(3HB) production of the native and constitutive operons. Together, these data suggest that the hybrid promoter performs a multiplicative, rather than an additive combination of the native and constitutive promoter designs. Unlocking the design rules of the hybrid promoter may have applicability that extends beyond the phaCAB operon. In instance, Li et al. 2012, suggest that engineered promoter clusters may be a useful metabolic engineering approach for increasing metabolite production. Additionally, model-guided optimisation of the PhaCAB pathway led to the generation of the constitutive operon design, which in combination with the hybrid promoter, represent an emerging family of rationally engineered P(3HB)-producing operons.

The broader project aim was to exploit non-recyclable waste as a low-cost carbon source that could be utilized by engineered E. coli tasked with the de novo synthesis of P(3HB). Industrial processing of non-recyclable mixed waste into solid and liquefied fractions of mixed cellulosic (~80%) and plastic (~20%) streams is currently possible [16]. However, these processes could be made more efficient through the use of natural and genetically engineered organisms that can both degrade and use these waste streams as a low-cost carbon source for the production of bioplastics. Beneficially, this approach may also result in the diversion of non-recyclable waste away from environmentally damaging activities such as landfill or incineration [3]. To this end, phaCAB-engineered E. coli were cultured in waste-media, which is analogous to industrially processed liquefied cellulosic waste; and from these cultures, P(3HB) was purified according to a modified sodium hypochlorite protocol, where waste-media cultures were filtered prior to centrifugation to remove residual waste material. Purified P(3HB) from each culture was depolymerized with recombinant phaZ1 into monomeric 3-hydroxybutyrate (3HB), which could then be detected as a yellow colour change when these samples were analysed with the β-Hydroxybutyrate colorimetric assay kit (S4 Supporting Information). PhaZ1-treated P(3HB) from E. coli carrying either the empty vector or native phaCAB operon resulted in a negligible color change, suggesting that little or no P(3HB) was produced in those cultures. Whilst, PhaZ1-treated P(3HB) from E. coli carrying either the constitutive phaCAB or hybrid phaCAB operon, depolymerized into 3HB, as indicated by a detectable, yellow color change. Additionally, flow cytometry analysis of Nile Red stained, [17,18] phaCAB-engineered E. coli from waste-media cultures, confirmed an increase in P-(3HB) content (Fig. 4).

In conclusion, our study used a forward-design approach to rationally engineer novel phaCAB operon designs that were experimentally validated to increase the production of P-(3HB) in genetically engineered E. coli. Additionally, we have described the utilization of non-recyclable waste as a low-cost carbon source for the production of P(3HB). With further development, these strategies, in combination with additional synthetic biology approaches [7,8], will further increase the efficiency and commercial viability of P(3HB) production.
Fig 4. Flow cytometry analysis of P(3HB) production in phaCAB-engineered E. coli from waste-media cultures. E. coli MG1655 transformed with either empty vector [EV], native [N], constitutive [C] or hybrid [H] phaCAB constructs were cultured in 5 ml of waste-media for 36 h at 37°C. P(3HB) content was assessed via flow cytometry analysis of Nile Red staining. (A) Representative forward scatter (FSC) and side scatter (SSC) contour plots. (B) Representative histogram (FL-5). (C) Normalized fluorescence of Nile Red stained phaCAB-engineered E. coli, from three independent experiments. Error bars, +/- the standard deviation. Student t-test, *P<0.05 and **P<0.001.

doi:10.1371/journal.pone.0117202.g004
Materials and Methods
Modeling
The P(3HB) synthesis model was constructed and simulated using the Simbiology toolbox of Matlab. The model is comprised of the glycolysis pathway, the tricarboxylic acid (TCA) cycle and the phaCAB synthetic pathway. Full details are provided as supplementary information (S1 Supporting Information).

Construct assembly
Empty vector (BBa_K608002), and native phaCAB (BBa_K934001) constructs were sourced from the 2013 distribution of the iGEM Registry of Standard Biological Parts (partsregistry.org). The constitutive phaCAB operon (BBa_K1149052) was generated via PCR, with the native phaCAB operon (BBa_K934001) as the template. Primers Pha_Fw 5’-cgcttcttagagtggtactgggaagggcctgg-3’ and BBa_G1005 5’-gtttcttcctgacggcctgtactagta-3’, were utilised to generate a PCR product containing the phaCAB operon but excluding the native promoter and RBS. The PCR product was cloned into the destination vector (BBa_K608002) to generate the final constitutive phaCAB operon construct. The hybrid phaCAB (BBa_K1149051) construct was generated in parallel to the constitutive phaCAB operon. The entire native phaCAB operon including the native promoter and RBS was cloned into the destination vector (BBa_K608002) to generate the final hybrid phaCAB operon construct. All constructs were generated using the iGEM submission backbone, pSB1C3 and all restriction digests utilized the standard iGEM prefix and suffix restriction sites. Full sequences are provided as supplementary information (S5 Supporting Information).

P(3HB) production
E. coli strain MG1655 carrying either empty vector, native phaCAB, constitutive phaCAB or hybrid phaCAB constructs were grown in LB media supplemented with 34 μg/mL Chloramphenicol (final concentration) for maintenance of plasmids. Cultures were incubated overnight at 37°C with shaking (200 rpm—Thermo Scientific MaxQ 6000). Resultant overnight cultures were diluted (1:200) into flasks containing either 1 liter of LB media, supplemented with 3% glucose (w/v) and 34 μg/mL Chloramphenicol or 600 ml of autoclaved waste-media (22 mM KH2PO4, 22 mM Na2HPO4, 85 mM NaCl, 0.1% [v/v] NH4Cl, 2 mM MgSO4, 0.1 mM CaCl2, 0.4% glucose, 2% [w/v] mixed non-recyclable waste—80% cellulosic, 20% plastic and dH2O), supplemented with 34 μg/mL Chloramphenicol. Cultures were subsequently incubated for 24 hours or 48 hours at 37°C with shaking (200 rpm—Thermo Scientific MaxQ 6000)

P(3HB) purification
This method is scaled down from an existing P(3HB) purification protocol [19]. Briefly, 1-liter production cultures were centrifuged at 4000 rpm (Beckman J2–M1) for 15 minutes. Waste-media cultures were filtered through Whatman filter paper in order to remove residual waste material before centrifugation. Post-centrifugation, bacterial cell pellets were re-suspended in phosphate-buffered saline (PBS), transferred into 50 ml tubes and centrifuged as described above. Cell pellets were washed with PBS and incubated for 30 minutes at room temperature in 1% (v/v in PBS) Triton-X 100. For the final purification of P(3HB), cells were centrifuged (4000 rpm—Beckman J2–M1 for 10 minutes), washed with PBS and incubated in aqueous sodium hypochlorite for 60 minutes at 30°C. The resultant purified P(3HB) granules were centrifuged (4000 rpm—Beckman J2–M1 for 10 minutes), washed with distilled water and dried.
overnight at 37°C. To determine the weight of the purified P(3HB), the weight of the 50 ml tube was subtracted from the combined weight of the 50 ml tube and P(3HB).

**Gene expression analysis with quantitative real-time PCR (qRT-PCR)**

To analyse phaCAB gene expression we adapted a previously described qRT-PCR protocol [7]. Briefly, 1 ml of each phaCAB-engineered E. coli strain was harvested after 24 h of growth in 100 ml LB, supplemented with 3% glucose (w/v) and 34 μg/mL Chloramphenicol. To stabilise RNA, 400 μl of RNA Protect Bacteria reagent (Qiagen, Hilden, Germany) was added to each cell pellet. Cell pellets were stored at −80°C until RNA extraction. ZR Fungal/Bacteria RNA Microprep kits (Zymo Research, CA, USA, #R2010) were used to harvest total RNA. To eliminate any remaining plasmid or genomic DNA, the samples were treated with RNase-free DNase I (NEB, Beverly, MA, USA) for 10 minutes, during the RNA extraction process. RNA purity and concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). cDNA synthesis from 500ng of total RNA was performed using the iScript Select cDNA synthesis kit (Bio-Rad, Richmond, CA, USA).

CDNA templates were used to determine gene expression levels by quantitative real-time PCR. Primers for phaA, phaB and phaC were designed according to previously published sequences [7]. To perform real-time quantitative PCR, the iQ SYBR green Supermix kit (Bio-Rad) was used and the reactions were run on a Mastercycler ep. realplex (Eppendorf, Hamburg, Germany). The PCR reaction conditions were: 3 minutes at 95°C and then 40 cycles of 15 seconds at 95°C, 1 minute at 60°C. To confirm the specificity of the PCR primers, a melting curve analysis was carried out. Data were analysed via the relative standard curve method and subsequently normalised to native phaCAB samples. Data were analysed from three biological replicates.

**Flow cytometry analysis**

Flow cytometry analysis of P(3HB) content was carried out as previously described [17,18,20]. Briefly, E. coli strain MG1655 carrying either empty vector, native phaCAB, constitutive phaCAB or hybrid phaCAB constructs were grown in 5 ml of waste-media (22 mM KH₂PO₄, 22 mM Na₂HPO₄, 85 mM NaCl, 0.1% [v/v] NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.4% glucose, 2% [w/v] mixed non-recyclable waste—80% cellulosic, 20% plastic and dH₂O), supplemented with 34 μg/mL Chloramphenicol. Cultures were subsequently incubated for 36 h at 37°C with shaking (200 rpm—Thermo Scientific MaxQ 6000). 1 ml of each overnight culture were centrifuged (8000 rpm—Eppendorf Minispin), washed with 1 ml PBS, and fixed with 35% Ethanol [v/v] at room temperature for 15 minutes. Post-fixation, cultures were centrifuged (8000 rpm—Eppendorf Minispin), re-suspended in 1ml PBS and stained with Nile Red (Sigma-Aldrich, MO, USA, #72485–100MG) to a final concentration of 20 μg/ml for 10 minutes on ice. Nile Red stained E. coli were diluted (1:100) into PBS and analyzed via flow cytometry. Around 65,000 cells per sample were loaded onto a BD-FACScan flow cytometer for detection of Nile Red staining (FL5, Ex 560, Em 610 nm) and data analysis from three biological replicates was carried out using FlowJo (vX 10.0.7r2) software. The background signal, as determined by the average geometric mean (FL-5) of Nile Red stained, empty vector transformed E. coli, was removed. Subsequently, these data were normalized to native phaCAB engineered E. coli.

**Detection of 3HB from PhaZ1-depolymerized P(3HB) with the β-Hydroxybutyrate (Ketone Body) colorimetric assay kit**

0.5 g of either purified P(3HB) or commercially sourced P(3HB) (Sigma-Aldrich, #363502–10G) were treated with 20μl PhaZ1 (0.36 μg/μl; BBa_K1149010) in 800 μl potassium phosphate buffer
(100 mM pH 7.4), shaking at 200 rpm (Thermo Scientific MaxQ 6000) for 5 hours at 37°C. PhaZ1-treated and untreated samples were diluted 13% and 1.3% (v/v) in β-Hydroxybutyrate assay buffer (100 mM Tris-HCl pH 8.4) and analysed with the β-Hydroxybutyrate (Ketone Body) colorimetric assay kit (Cayman Chemical, MI, USA) to detect the presence of 3HB, as indicated by a yellow colour change.

**Supporting Information**

**S1 Supporting Information. P(3HB) Modeling.** This supplementary file includes a 31-page document that contains complete details about the P(3HB) synthesis model we constructed to forward-design the phaCAB operons that increase P(3HB) production.

**S2 Supporting Information. P(3HB) Model.** MATLAB Simbiology P(3HB) model files.

**S3 Supporting Information. Nile Red staining of P(3HB).** *E. coli* MG1655 transformed with either empty vector, native, constitutive or hybrid phaCAB constructs were cultured for 24 h at 37°C and 200 rpm shaking (Thermo Scientific MaxQ 6000) in 5 ml LB media, supplemented with 3% glucose (w/v) and 34 μg/mL Chloramphenicol. Liquid cultures were streaked onto LB-agar plates supplemented with 3% glucose (w/v), 34 μg/mL Chloramphenicol and 0.5 μg/ml Nile Red staining (Sigma-Aldrich, MO, USA). Plates were incubated for up to 48 h at 37°C and imaged with a Fuji Film LAS-5000 imager set to 473 nm excitation laser and Cy5 emission filter.

**S4 Supporting Information. Qualitative analysis of 3-hydroxybutyrate (3HB) from PhaZ1-depolymerized P(3HB).** Purified P(3HB) from waste media cultured *E. coli* MG1655 carrying either empty vector, native phaCAB, constitutive phaCAB or hybrid phaCAB constructs were treated with or without the P(3HB) depolymerase, phaZ1. PhaZ1-treated and untreated samples were analysed with the β-Hydroxybutyrate (Ketone Body) colorimetric assay kit to detect the presence of 3HB, where a yellow colour change indicates the presence of 3HB.

**S5 Supporting Information. Engineered phaCAB operon sequences.**

**S6 Supporting Information. phaCAB gene expression levels in engineered E. coli.** *E. coli* MG1655 transformed with native [N], constitutive [C] or hybrid [H] phaCAB constructs were cultured in 5 ml LB media, supplemented with 3% glucose (w/v) for 24 h. Analysis of phaCAB gene expression was carried out using qRT-PCR and analyzed via the relative standard curve method. Experiments were carried out in triplicate and were normalized to native phaCAB–engineered *E. coli*. Error bars, +/- the standard deviation. Student t-test, +P < 0.05 and **P < 0.01.

**Acknowledgments**

The engineered operons were designed by 'Team Plasticity', comprising of seven undergraduate students from Imperial College London as part of a project in the 2013 International Genetically Engineered Machine Competition (iGEM). We would like to thank our colleagues in the Centre for Synthetic Biology and Innovation for your advice and also the iGEM Foundation for organizing the competition that instigated this work.
Author Contributions
Conceived and designed the experiments: R. Kelwick MK IB WC MHWC SF JP JS AJW KJ GBS R. Kitney PF. Performed the experiments: R Kelwick MK IB WC MHWC SF JP JS AJW KJ GBS. Analyzed the data: R. Kelwick MK IB WC MHWC SF JP JS AJW KJ GBS R. Kitney PF. Contributed reagents/materials/analysis tools: R. Kelwick MK IB WC MHWC SF JP JS AJW KJ GBS R. Kitney PF. Wrote the paper: R. Kelwick MK IB WC MHWC SF JP JS AJW KJ GBS R. Kitney PF. Supervised and co-ordinated this study: AJW KJ GBS R. Kitney PF.

References
1. Verlinden RA, Hill DJ, Kenward MA, Williams CD, Radecka I (2007) Bacterial synthesis of biodegradable polyhydroxyalkanoates. J Appl Microbiol 102: 1437–1449. PMID:17578408
2. Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biodegradable polyesters. Progress in Polymer Science 25: 1503–1555.
3. Harding KG, Dennis JS, von Blottnitz H, Harrison ST (2007) Environmental analysis of plastic production processes: comparing petroleum-based polypropylene and polyethylene with biologically-based poly-beta-hydroxybutyric acid using life cycle analysis. J Biotechnol 130: 57–66. PMID:17400318
4. Philip JC, Bartsev A, Ritchie RJ, Baucher MA, Guy K (2013) Bioplastics science from a policy vantage point. N Biotechnol 30: 635–646. doi:10.1016/j.nbt.2012.11.021 PMID: 23220474
5. Salehizadeh H, Van Loosdrecht MC (2004) Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. Biotechnol Adv 22: 261–279. PMID:14665402
6. Slater SC, Voige WH, Dennis DE (1988) Cloning and expression in Escherichia coli of the Alcaligenes eutrophus H16 poly-beta-hydroxybutyrate biosynthetic pathway. J Bacteriol 170: 4431–4436. PMID: 3049530
7. Hiroe A, Tsuge K, Nomura CT, Itaya M, Tsuge T (2012) Rearrangement of gene order in the phaCAB operon leads to effective production of ultrahigh-molecular-weight poly[(R)-3-hydroxybutyrate] in genetically engineered Escherichia coli. Appl Environ Microbiol 78: 3177–3184. doi: 10.1128/AEM.07715-11 PMID:22344649
8. Yang JE, Choi YJ, Lee SJ, Kang KH, Lee H, et al. (2014) Metabolic engineering of Escherichia coli for biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from glucose. Appl Microbiol Biotechnol 98: 95–104. doi:10.1007/s00253-013-5285-z PMID: 24113828
9. Raberg M, Voigt B, Hecker M, Steinbüchel A (2014) A closer look on the polyhydroxybutyrate- (PHB-) negative phenotype of Ralstonia eutropha PHB-4. PLoS One 9: e95907. doi:10.1371/journal.pone.0095907 PMID: 24787649
10. Kelwick R, MacDonald JT, Webb AJ, Freemont P (2014) Developments in the Tools and Methodologies of Synthetic Biology. Frontiers in Bioengineering and Biotechnology 2. doi: 10.3389/fbioe.2014.00083 PMID: 25610829
11. Kelly JR, Rubin AJ, Davis JH, Ajo-Franklin CM, Cumbers J, et al. (2009) Measuring the activity of BioBrick promoters using an in vivo reference standard. J Biol Eng 3. 4. doi: 10.1186/1754-1611-3-4 PMID:19298678
12. Weaver DS, Keskela IM, Mackie A, Paulsen IT, Karp PD (2014) A genome-scale metabolic flux model of Escherichia coli K-12 derived from the EcoCyc database. BMC Syst Biol 8: 79. doi: 10.1186/1752-0509-8-79 PMID: 24974895
13. Li M, Wang J, Geng Y, Li Y, Wang Q, et al. (2012) A strategy of gene overexpression based on tandem repetitive promoters in Escherichia coli. Microb Cell Fact 11: 19. doi: 10.1186/1475-2859-11-19 PMID:22305426
14. Le Meur S, Zinn M, Egli T, Thony-Meyer L, Ren Q (2013) Poly(4-hydroxybutyrate) (P4HB) production in recombinant Escherichia coli: P4HB synthesis is uncoupled with cell growth. Microbial Cell Factories 12, doi:10.1186/1475-2859-12-129 PMID: 24369062
15. Li ZJ, Cai L, Wu Q, Chen GQ (2009) Overexpression of NAD kinase in recombinant Escherichia coli harboring the phaCAB operon improves poly(3-hydroxybutyrate) production. Appl Microbiol Biotechnol 83: 935–947. doi: 10.1007/s00253-009-1943-6 PMID: 19357844
16. Jensen JW, Rønsch GØ, Antonsen SB (2013) Methods of processing municipal solid waste (msw) using concurrent enzymatic hydrolysis and microbial fermentation. Renescience A/S.
17. Lee JH, Lee SH, Yim SS, Kang KH, Lee SY, et al. (2013) Quantified high-throughput screening of Escherichia coli producing poly(3-hydroxybutyrate) based on FACS. Appl Biochem Biotechnol 170: 1767–1779. doi: 10.1007/s12010-013-0311-2 PMID: 23740474
18. Shakeri S, Roghanian R, Emtiazi G (2011) Comparison of intracellular polyhydroxybutyrate granules formation between different bacterial cell subpopulations by flow cytometry. Jundishapur Journal of Microbiology 4: 229–238.

19. Heinrich D, Madkour MH, Al-Ghamdi MA, Shabbaj II, Steinbuchel A (2012) Large scale extraction of poly(3-hydroxybutyrate) from Ralstonia eutropha H16 using sodium hypochlorite. AMB Express 2: 59. doi:10.1186/2191-0855-2-59 PMID: 23164136

20. Muller S, Bley T, Babel W (1999) Adaptive responses of Ralstonia eutropha to feast and famine conditions analysed by flow cytometry. Journal of Biotechnology 75: 81–97. PMID: 10617338

21. Quinn J, Beal J, Bhatia S, Cai P, Chen J, et al. (2013) Synthetic Biology Open Language Visual (SBOL Visual), version 1.0.0, BBF RFC #93.