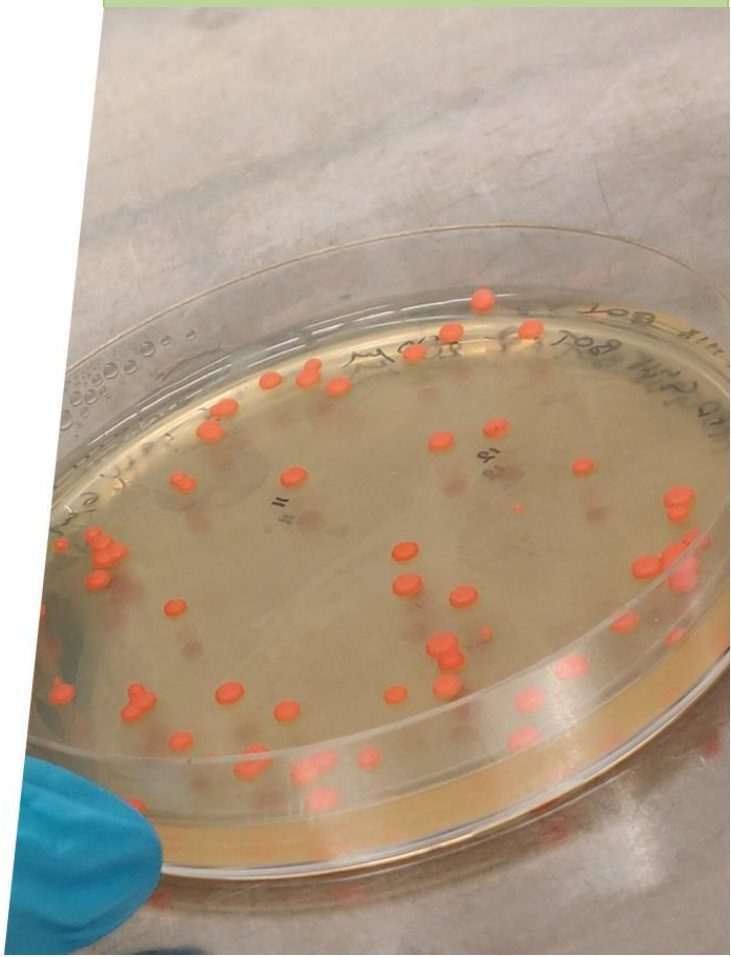
Lund University

Faculty of Engineering (LTH)

Engineering *Rhodosporidium toruloides* for the production of polyhydroxybutyrate

Master's Degree Project in Applied Microbiology

Tova Lindh



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Abstract

The increased use of plastics has become an environmental problem where plastics, due to the slow degradation, accumulate in the oceans and landfills. Although plastics are a very cheap and useful material with desirable properties and a wide variety of applications, they are primarily made from petroleum-based resources which are non-renewable and of fossil origin. An alternative to these non-renewable plastics are bioplastics, which can be both bio-based and bio-degradable. Poly-3-D-hydroxybutyrate (PHB) is a promising precursor for bioplastics and is produced by various microorganisms. PHB is bio-based and biodegradable, possesses the characteristics of thermoplastics, and its mechanical properties are comparable to the chemical properties of polypropylene. PHB can be produced from acetyl-CoA in a three-step procedure involving the enzymes acetyl-CoA acetyltransferase, acetoacetyl-CoA reductase and polyhydroxyalkanoate synthase (PHB synthase). The enzymes are encoded by the genes PhaA, PhaB and PhaC respectively. A non-conventional oleaginous yeast is believed to potentially be a good producer of PHB as oleaginous yeasts are known to possess efficient routes for acetyl-CoA biosynthesis.

In the present study, the codon-optimized genes PhaA1, PhaB1 and PhaC1 from *Cupriavidus necator* were successfully integrated into the genome of the oleaginous yeast *Rhodosporidium toruloides* using a recently developed electroporation protocol through random integration. Two clones were proven to produce PHB and further characterized. PHB production from glucose was demonstrated in shake-flasks experiments, where the best strain resulted in a PHB titer of 77 mg PHB/L, PHB yield of 3.7 mg PHB/g glucose and PHB yield on biomass of 0.0082 g PHB/g CDW. This study demonstrates that the codon-optimized genes are functional in the non-conventional oleaginous yeast used and that the production of PHB in the recombinant strains is possible.

Popular science summary – Bioplastics from micro-factories

A commonly used material in today's society is plastics, which is a very cheap material with a lot of applications due to its many desirable characteristics. Some of these however, have downsides, such as the slow degradation which is a problem when the plastics end up in nature. Another issue is that most of the plastics produced today originate from fossil resources, which make them non-renewable. An alternative material that has gained a lot of attention in recent years due to its potential to solve both of these problems, is bioplastics: a type of plastics that can be produced from renewable sources and degraded e.g., in composts.

One biopolymer that is of high interest for production of bioplastics is called polyhydroxybutyrate (PHB). PHB has characteristics that are similar to petroleum-based plastics and can be produced by different microorganisms. To produce PHB in microorganisms three genes are needed. The goal of this project was to insert these genes in a yeast that normally does not produce PHB and thereby turn the yeast cells into small micro-factories for biopolymers. The yeast that was chosen for this project is believed to have many desirable characteristics that will allow it to make higher amounts of PHB than other microbes. To make this possible, the genes were optimized so that they would be understood by the yeast and then bought from a company that make synthetic genes. The genes were delivered as small circular fragments of DNA, called plasmids. The three genes required for PHB production, together with one antibiotic resistance gene were then assembled together into one large piece of circular DNA (plasmid), by cutting-and-pasting the different pieces together using enzymes. To insert the genes in the yeast, the outer layer of the yeast cells (their "skin") was weakened using a chemical treatment and an electric shock so that the new DNA could easier be transferred to the inside of the cell. Once inside, the DNA has to be integrated into the genome of the cell to be functional. The yeast has a DNA-repairing system that helps it survive damages to the DNA, that can take the new DNA and integrate it in the chromosome during the repairing. To find which cells have properly put the new DNA in its genome (three PHB genes and one antibiotic resistance gene), the cells are grown in presence of the antibiotic, and only the cells with the new DNA will survive.

After these genetically modified yeast cells had been constructed, it was time to test if they worked as small micro-factories that produce PHB. The cells were cultivated for 3 days and samples to analyze if PHB was produced were taken. The cells were also evaluated for how fast they grew and consumed glucose. In the end, a yeast that could produce PHB was found. Future work can now try to make these micro-factories better and more efficient.

1. Introduction

The usage of plastics in our society has increased since the 1950s and it has become an essential material utilized globally. According to the UN Environment program the global production has on average increased by 9% per year since 1950. In 2015 the global plastics production was around 388 million tons, and this industry is a major economic actor with a revenue corresponding to approximately 3% of the global economy. However, this large production has impacts on the environment: in the same UN Environment program report from 2018, it is stated that around 80% of all plastics ends up in the oceans, corresponding to between 8 – 12 million tons per year. The slow degradation of plastics also results in an accumulation of plastics in the landfills or the natural environment, causing plastics pollution (UN Environment, 2018).

Plastics are a very cheap material with desirable properties like barrier functions and high durability. The wide variety of application areas and properties that plastics possess makes it a useful material; however, it is primarily made from petroleum-based resources which are non-renewable and of fossil origin. To solve this problem, the usage of bioplastics has gained a lot of attention over the last years as an alternative to petroleum-based plastics. Bioplastics are categorized into three groups: bio-based, bio-degradable or a combination of both. One example is polyhydroxyalkanoates (PHAs) that are biopolymers with plastic-like properties and belonging to the third group of bioplastics (Ashter, 2016).

Polyhydroxyalkanoates (PHAs) are bio-based and biodegradable polyesters which can be produced by bacteria through fermentation of sugars and lipids. In general, PHAs are linear biopolymers that are non-toxic, biocompatible and insoluble in water (Alcântara, et al., 2020). PHA polymers can be used as bioplastics in packaging material, disposable medical tools, or food service materials and in some paints. (Ahmed, et al., 2018). Poly-3-D-hydroxybutyrate (PHB) is the most common and well-studied type of PHAs and is produced by various microorganisms (Choi and Lee, 1997). The structure of PHB can be seen in *Figure 1* below, which also illustrates the chirality of the molecule.

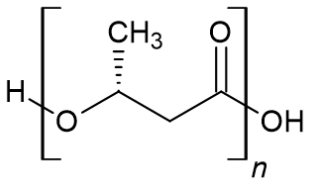

Figure 1: Chemical structure of Poly-3-D-hydroxybuturate (PHB), n times forming the polymer.

PHB is naturally produced in a wide range of microorganisms as a carbon and energy storage (Lugg, et al., 2008). The biopolymer is produced and accumulated in the cells when nutrients such as nitrogen or sulfate are limited, and the carbon source is present in excess (Kocharin, et al., 2012). PHB is stored as granules in the cytosol of the cells. When nutrients are present again, the PHB granules can be consumed by the microorganism again. Characteristics of PHB that make it an interesting polymer for use in bioplastics are its high melting temperature, low permeability to O_2, H_2O and CO_2 and high degree of crystallinity. PHB is also interesting as a replacement for synthetic petroleum-based polymers since it possesses the characteristics of thermoplastics, its mechanical properties are comparable to the chemical properties of polypropylene and it is biodegradable without the generation of toxic by-products (Choi and Lee, 1997; Rajan, et al., 2017). Other interesting applications of PHB can be found in the medical and pharmaceutical industry, due to its biocompatibility, lack of

any inflammatory response in the human body and its slow degradation process (Rajan, et al., 2017 and Peña, et al., 2014).

Some natural producers of PHB are *Cupriavidus necator* as well as several species of *Pseudomonas*, *Bacillus* and *Azotobacter* (Choi and Lee, 1997; Olivera, 2001; Peña, et al., 2014; Centeno-Leija, 2014). PHB has also been successfully produced by recombinant strains of e.g., *Escherichia coli* or *Saccharomyces cerevisiae*, expressing the genes for PHB production from *C. necator* or *Azotobacter vinelandii* (Peña, et al., 2014 and Sandström, et al., 2015). In the review of Peña et al. from 2014, a comparison was made between different PHB producers on different carbon sources: *C. necator* DSM545 produced 0.76 g PHB/g CDW grown on glucose (Mozumder, et al., 2014) and *E. coli* recombinant GCSC 6576 produced 0.81 g PHB/g CDW grown on whey (Kim, 2000). One example of *S. cerevisiae* is the engineered strain TMB 4425 which has been reported to produce 0.73 g PHB/L corresponding to 0.164 g PHB/g CDW, while cultivated anaerobically on xylose (Portugal-Nunes, et al., 2017). The idea behind recombinant strains is to see if it is possible to produce more PHB than the natural producers and to use new organisms suitable for industrial scale growing on complex substrates.

PHB is produced from the intermediate acetyl coenzyme A (acetyl-CoA) in a three-step procedure involving the enzymes acetyl-CoA acetyltransferase, acetoacetyl-CoA reductase and polyhydroxyalkanoate synthase (PHB synthase). The enzymes are encoded by the genes PhaA, PhaB and PhaC respectively. The PHB metabolic pathway is illustrated in *Figure 2* below with metabolite structure, gene names, involved enzymes and cofactors. Acetyl-CoA acetyltransferase catalyzes the condensation of two Acetyl CoA to one acetoacetyl-CoA, acetoacetyl-CoA reductase then reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA with the cofactor NADPH, and PHB synthase catalyzes the final polymerization step of (R)-3-hydroxybutyryl-CoA into PHB (Portugal-Nunes, et al., 2017).

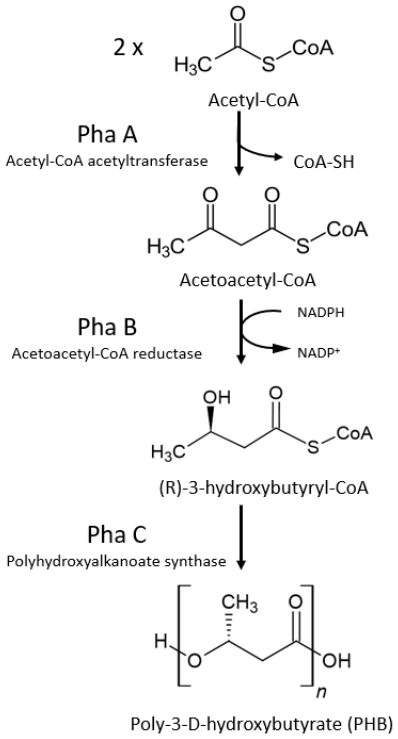

Figure 2: The PHB metabolic pathway with metabolite structure, gene names, enzymes and cofactors.

To make a sustainable and profitable production of PHB, high yields and productivities are required (Lugg, et al., 2008). Choi and Lee states that the final PHB price per kg can be higher than for petroleum-based alternatives as consumers are likely to pay a little extra for biodegradable alternatives, although the difference cannot be too high (Choi and Lee, 1997). The precursor for PHB in the pathway described above is acetyl-CoA and to improve the PHB production, species that are known to produce a lot of acetyl-CoA are interesting candidates for use as host organisms. Oleaginous yeasts are known to accumulate substantial amounts of lipids, quantifying more than 20% of their biomass (Vorapreeda, et al., 2012). Oleaginous yeasts are known to have efficient fluxes towards acetyl-CoA production and indications of possessing additional routes for acetyl-CoA biosynthesis compared to non-oleaginous yeast (Vorapreeda, et al., 2012). *Rhodosporidium toruloides* is an oleaginous yeast which has increased in interest during the last years for the potential of using it as an industrial yeast for chemicals, fuels, food and pharma (Park, et al., 2018). *R. toruloides* is able to grow to high cell density and utilize a wide range of carbon and nitrogen substrate and is a natural producer of carotenoids, lipids and some enzymes that are industrially relevant. Carotenoids provide antioxidant properties and make the cells appear red/pink in color upon carotenoid accumulation, and thus *R. toruloides* is sometimes nicknamed “pink yeast”. The lipids, mainly triacylglycerol (TAG), are produced as a carbon and energy storage during nutrient limitations. *R. toruloides* can produce and accumulate lipids up to 65% of the cell dry weight (CDW) (Park, et al., 2018). *R. toruloides* is a non-conventional yeast belonging to basidiomycota phylum which means that it can form spores and reproduce sexually, and it can also reproduce through non-sexual reproduction (Kurtzman, et al., 2011).

Genetic tools for engineering of *R. toruloides* are crucial for further usage of *R. toruloides* as an industrial yeast and, as more methods are being developed and made available, the more promising this yeast becomes as a production host in industrial applications (Park, et al., 2018). In *R. toruloides* double stranded breaks in the DNA are mainly repaired through non-homologous end-joining (NHEJ) and not through homologous recombination, which is regarded as the main reason to the low gene targeting efficiency (Koh, et al., 2014). Homologous recombination is possible in *R. toruloides,* but the efficiency is very low (Sun, et al., 2017). NHEJ results in random integration events, which often not is preferred for engineering purposes. It has however been shown that targeted integration can be improved by disabling the NHEJ system (Koh, et al., 2014).

A natural isolate of *R. toruloides* called BOT-A2, isolated from the botanical gardens in Gothenburg Sweden has been shown to produce a lot of lipids (~40%; Qvirist, Vazquez Juarez and Andlid, manuscript in preparation) and is believed to potentially be a good producer of PHB. Recently a transformation protocol has been developed in-house by Brink and Darr (unpublished) which make genetic engineering through electroporation of *R. toruloides* BOT-A2 possible. Sandström et al. (2015) proved that PHB can be produced in yeast (*S. cerevisiae*) through codon-optimized expression of the genes PhaA1, PhaB1 and PhaC1 from *C. necator* and a similar strategy was used in the present study. In this proof-of-concept study, I aimed at constructing a recombinant *R. toruloides* strain that carry codon-optimised genes for production of PHB from acetyl-CoA and at evaluating PHB production from glucose.

2. Material and Methods

2.1 Plasmids, strains and media

Plasmids and yeast strains used in this study are listed in *Table 1* below. For the construction of pUC57-PhaAB and pUC57-PhaC the genes PhaA, PhaB and PhaC from *C. necator* were codon optimized for *R. toruloides* and synthesized at GenScript (Leiden, The Netherlands). Amino acid sequences for *C. necator* PhaA (UniProt: G0ETI6), PhaB (UniProt: G0ETI7), PhaC (UniProt: G0ETI5) were codon optimized using the OPTIMIZER tool (http://genomes.urv.es/OPTIMIZER/; Puigbo et al 2007) and the *R. toruloides* codon usage table from Codon Usage Database (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=5286). This database has previously been successfully used to codon optimize GFP for *R. toruloides* (Liu et al, 2013). All the coding sequences were designed to be under the control of constitutive glycolytic promotors previously evaluated in *R. toruloides*: RtGPD1p, RtFBA1p and RtPGI1p (Wang, et al., 2016). Eukaryotic terminators that have previously been used for engineering *R. toruloides* were also used: AtNOSt (from plasmid NM1-5S-tRNA-SgH), NcBetaTubt (from plasmid NM9-SpCas9-NLS3), RgTUB2t (from plasmid NM9-SpCas9-NLS3) originate from Schultz et al., (2019); RgTUB2t (GenBank: KX377639) originate from Zhang et al., (2016). The codon optimized KanMX (from NM9-SpCas9-NLS3; Schultz et al., (2019)) was also used.

E. coli strain NEB5α (New England Biolabs) was used for sub-cloning of plasmid DNA. Lysogeny Broth (LB) medium containing 10 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl, pH 7.0 was used for culturing *E. coli*. 15 g/L Agar-Agar was added for plates. 100 µg/mL ampicillin was added to the LB when required. Bacterial transformants were selected on LB agar plates containing ampicillin as selection pressure, for 16 h at 37 °C. Transformants that were successfully verified by colony PCR were cultivated at 37°C for 16h in LB with ampicillin and stored in 25% (v/v) glycerol stocks at -80 °C. Glycerol stocks were recovered by plating on LB agar plates with ampicillin before cultivation in liquid LB medium supplemented with ampicillin for 16 h at 37 °C on a rocking table.

Yeast strains (*R. toruloides* and *S. cerevisiae*) were recovered from 25% (v/v) glycerol stocks stored at -80 °C by streaking on solid Yeast Peptone Dextrose (YPD) plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L Agar-Agar, for 2 – 3 days at 30 °C. 200 µg/mL geneticin (G418) was added to YPD when required. Yeast Nitrogen Base (YNB) medium containing 6.7 g/L Yeast Nitrogen Base without amino acids (Becton, Dickinson and Company, USA), potassium buffer (2.299 g/L K_2HPO_4, 11.83 g/L KH_2PO_4, pH 5.5) and supplemented with 20 g/L glucose was used for some of the yeast cultivations. Yeast transformants was cultivated overnight in YPD and geneticin (30°C) and stored in 25% (v/v) glycerol stocks stored at -80 °C.

All chemicals were purchased from Sigma-Aldrich (Missouri, USA) if not otherwise stated and restriction enzymes were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Table 1: Plasmids and strains used in this study. Abbreviations: Rt = Rhodosporidium toruloides; Nc = Neurospora crassa; Rg = Rhodotorula graminis; At = Agrobacterium tumefaciens; Av = Allochromatium vinosum; Sc = Saccharomyces cerevisiae.

Plasmids	**Relevant genotype**	**Reference**
pUC57	Standard *E. coli* cloning vector derived from pUC19	Thermo Fischer (SD0171); pUC19 (Yanisch-Perron et al 1985)

pDB21	pUC57; -RtGPD1p-KanMX(Rt codon opt)- NcβTubt [KanMX cassette cloned from NM9-SpCas9-NLS3]	Brink, (unpublished); NM9-SpCas9-NLS3 parts originally from Schultz et al., (2019)
pUC57-PhaC	pUC57; RtFBA1p-PhaC(Rt codon opt)-RgTUB2t	Brink, (unpublished)
pUC57-PhaAB	pUC57; RtPGI1p-PhaA(Rt codon opt)-AtNOSt; RtPGK1p-PhaB(Rt codon opt)-CMV35St	Brink, (unpublished)
pDB30	pUC57-PhaC; RtGPD1p-KanMX(Rt codon opt)-NcβTubt	This study
pDB31	pUC57-PhaAB; RtFBA1p-PhaC(Rt codon opt)-RgTUB2t-GPD1p-KanMX(Rt codon opt)-NcβTubt	This study
***E. coli* strains**	**Relevant genotype**	**Reference**
NEB5α	*Escherichia coli* K-12*; endA1, recA1, hsdR17, ΔlacZ58(M15), fhuA2*	Hanahan, D., (1985) and Anton et al., (2016)
NEB5α-pDB30	NEB5α; pDB30	This study
NEB5α-pDB31	NEB5α; pDB31	This study
Yeast strains	**Relevant genotype**	**Reference**
S. cerevisiae TMB 4425	CEN.PK2-1C; *gre3Δ; his3::pPGK1-XKS1-tPGK1, HIS3; tal1::pPGK1-TAL1-tPGK1; tkl1::pPGK1-TKL1-tPGK1; rki1::pPGK1-RKI1-tPGK1; rpe1::pPGK1-RPE1-tPGK1; ura3::YIpDR7; leu2 (pPGK1-XYL2-tPGK1; URA3); leu2::YIpAGS3 (pTEF1-PhaA(Sc codon opt)-tTEF1; pGPM1- AvPhaB(Sc codon opt)-pGPM1; pTPI1-PhaC1(Sc codon opt)-tTPI1; LEU2)*	de las Heras, et al., (2016)
R. toruloides BOT-A2	Wild type (natural isolate)	Qvirist, et al., (unpublished)
R. toruloides TMB DB061	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB062	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB063	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB064	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB065	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB066	BOT-A2; pDB31 (random integration)	This study

R. toruloides TMB DB067	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB068	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB069	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB070	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB071	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB072	BOT-A2; pDB31 (random integration)	This study
R. toruloides TMB DB073	BOT-A2; pDB31 (random integration)	This study

2.2 Molecular biology and plasmid construction

The synthesized plasmids pUC57-PhaAB and pUC57-PhaC were each transformed into NEB5α competent *E. coli* strains and selected on LB plates supplemented with ampicillin for 16 h at 37 °C. The plasmids pUC57-PhaAB and pUC57-PhaC were respectively extracted from the obtained transformants using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). The plasmids were sent for sequencing to Eurofins for confirmation of the correct sequences.

The geneticin resistance gene (KanMX) with promoter and terminator was amplified by PCR from pDB21 with primers containing restriction sites for HindIII and PaeI respectively. The fragment was amplified with Phusion polymerase (Thermo Fisher Scientific, USA) using the following PCR program: initial denaturation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 10 s, annealing at 65.4 °C for 30 s and elongation at 72°C for 41 s, and a final elongation step for 10 min at 72°C. The primers used were RtGPD1p_1F_PaeI and NcBtubt_280R_HindIII and the sequences can be found in *Table 2* below. All oligonucleotides used for PCR are listed in *Table 2.* The PCR fragment was purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The PCR product was digested with the enzymes HindIII and PaeI to create sticky ends and DpnI to get rid of any background plasmids (PCR templates). 200 ng amplified DNA was used, and the digestion followed the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA), incubation for 30 minutes at 37 °C and heat inactivation for 10 minutes at 80 °C. The plasmid pUC57-PhaC was linearized using the restriction enzymes HindIII and PaeI. 1000 ng of plasmid DNA was used together with the same conditions as described above for the PCR product. The PCR product, digested PCR product and digested plasmid were verified using agarose gel electrophoresis. The fragment was ligated into the linearized plasmid using T4 ligase (Thermo Scientific, USA) according to the manufacturer's instructions, incubation for 1 h at room temperature and the molar ratio of 3:1 insert to backbone. The ligated product was then transformed into NEB5α competent *E. coli* (prepared according to Inoue et al 1990) and selected on LB plates supplemented with ampicillin for 16 h at 37 °C. *E. coli* colony PCR was performed for verification on 16 of the transformants: colonies were boiled at 97°C in 25 µl sterile water for 5 min and PCR was performed using the primers RtGPD1p_1F_phos and NcBtubt_301R_phos and DreamTaq polymerase (Thermo Fisher Scientific, USA). Verified colonies were cultivated in 5 mL LB supplemented with ampicillin in 50 mL conical centrifuge tubes for 16 h at 37 °C on a rocking table, and plasmids were extracted with GeneJET PCR Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). The final plasmid was sent to Eurofins for sequencing and when confirmed store in -80°C and named pDB30.

Table 2: Primers used in this study and their sequences.

Name	Amplification target	Sequence (5' to 3')
RtGPD1p_1F_PaeI	KanMX cassette	TATGCATGCCTGCAGAACTACGCCCTCG
NcBtubt_280R_HindIII		AATAAGCTTGCCAGCAGTAGACACTTGGAAT
RtGPD1p_1F_phos	KanMX cassette	[PHO]CTGCAGAACTACGCCCTC
NcBtubt_301R_phos		[PHO]GCCAGCAGTAGACACTTGG
RtFBA1p_pUC57PhaC_F_phos	KanMX and PhaC cassette	[PHO]CGGCCAGTGAATTCCTCTGCT
NcBtubt_301F_phos		[PHO]GCCAGCAGTAGACACTTGG
RtKanMX_1F	KanMX	ATGGGCAAGGAGAAGACGCA
RtKanMX_810R		CTAGAAGAACTCGTCGAGCATGA
PhaA_55F	PhaA	GGCTCGCTCGCCAAGATCCC
PhaA_1111R		GAGGCGAGGCCCTTCTTGGC
PhaB_148F	PhaB	GCCCTCGGCTTCGACTTCGT
PhaB_715R		ATGTGGAGGCCGCCGTTGAG
RtPhaC_46F	PhaC	TCGCAGCCGTTCAAGTTCAC
RtPhaC_1550R		GTGCGAGCGCTTGTTCTTGG
M13uni-43	Backbone pUC57	AGGGTTTTCCCAGTCACGACGTT
PhaB_449F	PhaB	AGACCAACTACTCGACCGC

From pDB30, the fragment containing the genes PhaC and KanMX and their respective promoters and terminators was amplified by PCR with phosphorylated primers (for later blunt cloning). The fragment was amplified with Phusion polymerase (Thermo Scientific, USA) using the following PCR program: initial denaturation at 98°C for 30 s, 30 cycles of denaturation at 98°C for 10 s, annealing at 63.3 °C for 30 s and elongation at 72°C for 1 min and 38 s, and a final elongation step for 10 min at 72°C. The primers used were RtFBA1p_pUC57PhaC_F_phos and NcBtubt_380F_phos. The PCR fragment was purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) and verified using agarose gel electrophoresis. The plasmid pUC57-PhaAB was linearized using the restriction enzyme SmaI, and phosphates in the linearized backbone were removed with Fast AP (Alkaline Phosphatase) to minimize the chance of self-ligation. 1000 ng of plasmid DNA was used, and the digestion followed the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA), incubation for 10 minutes at 37 °C and heat inactivation for 15 minutes at 65 °C. The phosphorylated fragment was ligated with blunt ends into the linearized plasmid using T4 ligase (Thermo Scientific, USA) according to the manufacturer's instructions, incubation for 1 h at room temperature and a molar ratio of 3:1 insert to backbone. The ligated product was then transformed into NEB5α competent *E. coli* and selected on LB plates supplemented with ampicillin for 16 h at 37 °C. Colony PCR was performed for verification on 32 of the transformants using the primers RtKanMX_1F and RtKanMX_810R. The

final plasmid was sent to Eurofins for sequencing and when confirmed saved and named pDB31. A schematic overview of the plasmid construction work is illustrated in *Figure 3* below.

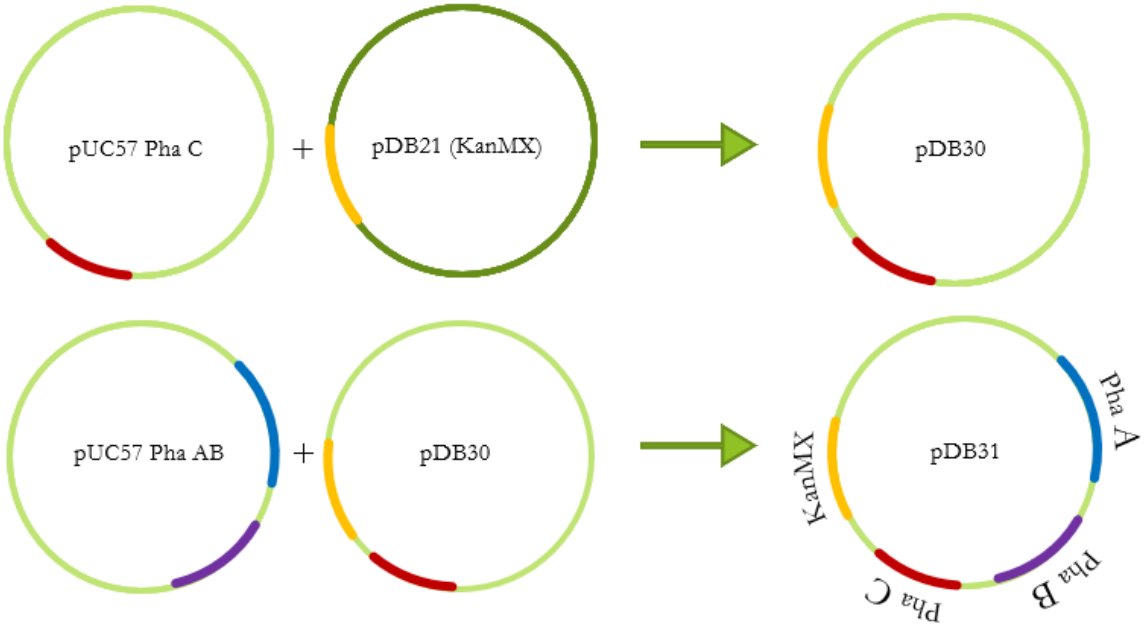

Figure 3: Schematic overview of plasmid construction.

To linearize pDB31 for transformation in *R. toruloides* (which requires ~1µg DNA/kb sequence) the 14.5 kb plasmid was digested in an upscaled digestion reaction, using the restriction enzyme SspI. A total of 35 000 ng plasmid DNA were digested by splitting the reaction into 7 tubes with 5000 ng DNA and 5 µl SspI. The digestion was performed according to the manufacturer's instructions (Thermo Scientific, USA), incubation for 30 minutes at 37 °C and heat inactivation for 5 minutes at 65 °C. The linearized plasmids were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), where 55 % yield loss over the purification column occurred.

2.3 *Rhodosporidium* strain engineering

R. toruloides BOT-A2 was transformed with the linearized pDB31. Cultures of the yeast strains were grown in liquid YPD medium overnight at 30 °C and 180 rpm in an orbital shaker, then reinoculated to an optical density (620 nm; OD_{620}) of 0.5 in 25 mL YPD (in 250 mL baffled shake flasks) and grown for additionally 4 h, when preparing the strains for transformation. The yeast cells were transformed using the recently in-house developed electroporation protocol (Brink and Darr, unpublished). In short, cells were harvested when OD_{620} ~ 1.5 by centrifugation at 3220 g for 5 min in a 50 mL conical centrifuge tube and decanting the supernatant. The cells were washed in 10 mL TMLSD buffer (10 mM Tris-HCl buffer at pH 8.0, 1 mM $MgCl_2$, 100 mM lithium acetate, 270 mM sucrose, 5 mM dithiothreitol) and then resuspended in 10 mL TMLSD and incubated 1 h at room temperature. The cells were centrifuged again and washed two times in 10 mL TMS buffer (10 mM Tris-HCl buffer at pH 8.0, 1 mM $MgCl_2$) before one wash with 1 M cold sorbitol. Then the cells were resuspended in cold sorbitol to OD_{620} = 100 and 50 µL was aliquoted in each electroporation cuvette. Linear and PCR purified DNA, ~1000 ng/kb, was added. Electroporation was done with the following settings: pulsing at 1.9 kV, resistance 400 Ω and capacitance 25 µF (Bio-Rad Gene Pulser™, Bio-Rad Laboratories, Inc, Hercules, CA, US). After

electroporation 950 µL cold YPD was added to the cuvette before transferred to a micro centrifuge tube and incubated at 30 °C for 2 h on a rocking table. The yeast transformants were selected by plating on YPD agar plates supplemented with geneticin and incubated at 30 °C for 2 – 4 days. Due to the random integration, each colony had a different genotype and therefore represents a different strain. Transformed strains TMB DB061 to TMB DB073 were validated by amplifying several parts of the integrated cassette by yeast colony PCR, using extracted genomic DNA as template. The genomic DNA was extracted by boiling a small amount of cells in 15 µL 0.02 M NaOH at 99 °C for 10 minutes. All fragments were amplified with DreamTaq polymerase (Thermo Scientific, USA). The PCR programs with respective primer pairs that were used are presented in *Table 3* below.

Table 3: Yeast colony PCR assays for gene validation of transformants. Primer pairs and programs used. Due to how R. toruloides BOT-A2 randomly integrates the recombinant DNA during the transformation, the integration loci were unknown, and primers were designed to only anneal in the recombinant DNA.

Assay	Primer pair	PCR program
PhaA	PhaA_55F/PhaA_1111R	Initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 65.4 °C for 30 s and elongation at 72°C for 1 min, and a final elongation step for 10 min at 72°C.
PhaB	PhaB_148F/PhaB_715R	Initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 64.3 °C for 30 s and elongation at 72°C for 1 min, and a final elongation step for 10 min at 72°C.
PhaC	RtPhaC_46F/RtPhaC_1550R	Initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60.2 °C for 30 s and elongation at 72°C for 1 min, and a final elongation step for 10 min at 72°C.
PhaA promotor	M13uni-43/PhaA_1111R	Initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 65.7 °C for 30 s and elongation at 72°C for 2 min and 41 s, and a final elongation step for 10 min at 72°C.
PhaB terminator and PhaC promotor	PhaB_449F/RtPhaC_1550R	Initial denaturation at 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58.7 °C for 30 s and elongation at 72°C for 3 min and 40 s, and a final elongation step for 10 min at 72°C.

The PCR fragments were verified using agarose gel electrophoresis. Some of the PCR products were sent to Eurofins for sequencing for confirmation of the correct sequences.

2.4 Aerobic cultivation in shake flasks for screening of PHB production

Pre-cultures of yeast strains (parental and engineered *R. toruloides* as well as *S. cerevisiae* TMB4425 engineered with a PHB pathway) were started by inoculating single colonies in 5 mL YPD medium in 50 mL conical centrifuge tubes incubated for 16 h at 30 °C and 180 rpm in an orbital shaker. The cells were inoculated in 25 mL YPD medium in 250 mL baffled shake flasks to a starting OD_{620} of 0.5 and grown at 30 °C and 180 rpm in an orbital shaker for 72 h. Endpoint measurements (72 h) of OD_{620} using Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, Uppsala, Sweden) were done in technical duplicates. Endpoint samples (72 h) for PHB quantification were also taken. Cultivations were performed in biological duplicates. For all shake flasks experiments, well-aerated baffled shake flasks were used.

2.5 Aerobic cultivation in shake flasks for characterization of the top *R. toruloides* PHB producers

Pre-cultures of yeast strains were started by inoculating single colonies in 25 mL defined YNB medium in 250 mL baffled shake flasks incubated for 24 h at 30 °C and 180 rpm in an orbital shaker. A desired volume of the cells was harvested and washed with sterile MQ water by centrifugation in 50 mL conical centrifuge tubes for 5 minutes at 6100 g before resuspended in 10 mL defined YNB medium. The resuspended cells were inoculated to a total volume of 100 mL defined YNB medium in 1 L baffled shake flasks to a starting OD_{620} of 0.5 and grown at 30 °C and 180 rpm in an orbital shaker for 72 h. Samples for OD_{620}, glucose, CDW, microscopy, and PHB quantification was taken during the cultivations. At the end of the cultivations the pH was measured. Samples for OD_{620}, glucose and CDW was performed in technical duplicates. Cultivations were performed in biological duplicates.

2.6 Analysis of biomass and glucose

Filters (0.45 µm pore size membrane filters; Pall Corporation, New York, USA) were pre-dried in a microwave oven at 350 W for 4 minutes, equilibrated to room temperature in a desiccator over night before weighed. Samples were regularly taken for cell dry weight determination by vacuum filtrating a known volume (1.0 to 5.0 mL) of the culture through a pre-weighed filter. The filters were washed with MQ water, dried in a microwave oven at 350 W for 8 minutes and weighed after equilibration to room temperature in a desiccator.

Samples for glucose concentrations were taken throughout the cultivations. The samples were centrifuged for 5 minutes at 13000 g and the supernatant was transferred to fresh micro centrifuge tubes and stored at -20 °C before being analyzed. A Waters HPLC system (Milford, USA) equipped with Aminex HPX-87H ion exchange column (Bio-Rad, USA) operating at 60 °C with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.6 mL/min was used. Glucose was detected with a refractive index detector (Waters model 2414; Milford, MA, USA). The glucose concentrations were calculated using an external seven-point calibration curve (10 g/L down to 0.1 g/L).

2.7 PHB quantification

PHB quantification relies on the quantitative hydrolysis of PHB to crotonic acid catalyzed by hot concentrated sulfuric acid (Law and Slepecky, 1961). Since PHB is stored intracellularly, cell pellets were collected and subjected to the hot acid hydrolysis. A total culture volume of 4 mL was harvested by two serial centrifugations for 5 minutes at 6100 g each in a 2 mL micro centrifuge tube and the supernatant was discarded through aspiration (FTA-1, Biosan, Latvia). The pellet was washed two times with 1.0 mL sterile MQ water, resuspended by pipetting up and down and vortexing, centrifuged for 5 minutes at 6100 g, and the supernatant was aspirated and discarded. If the pellet was not analyzed on the same day, it was stored at -20 °C and then recovered from the freezer. The pellet was carefully resuspended in 500 µL H_2SO_4 (95 – 97 %) by pipetting and the tubes were transferred to a heat block

(Grant QBD1, Grant Instruments, UK), and incubated at 95°C for 1 h with open lids. The samples were left to cool down (for ca 5-10 min) and were then diluted 10 times in MQ water. The diluted samples were centrifuged for 5 minutes at 13200 g to separate particles formed during the hydrolysis. Around 800 µL of the supernatant was transferred to vials for analysis by HPLC, using the same system as detailed above. The same conditions were used except for the detector where a UV/Visible detector (Waters model 2489; Milford, MA, USA) was used to measure the absorbance at 210 nm. Crotonic acid concentrations were calculated using an external calibration curve of crotonic acid (10 points; 1 g/L-0.001 g/L). Commercially available PHB (#363502, Sigma-Aldrich) was used as method control in known concentrations and were processed in the same way as the samples.

3. Results

3.1 Plasmid construction and strain engineering

Expression of the genes required for PHB production and product formation in *R. toruloides* was evaluated in this study. Synthesized plasmids (pUC75-PhaAB and pUC67-PhaC) containing a *R. toruloides* codon-optimized PHB pathway genes were subcloned in *E. coli,* extracted, sequenced and validated. They were used to construct the plasmids pDB30 carrying the codon optimized genes for PhaC and KanMX, and pDB31 carrying the codon optimized genes for PhaABC and KanMX that were also verified through colony PCR and further sequencing of the positive clones.

R. toruloides BOT-A2 was transformed with the linearized pDB31 (random chromosomal integration) and selected for growth on YPD agar plates supplemented with geneticin. 13 transformants representing different strains (each with different random integration locus) were obtained. While all transformants had the geneticin resistance phenotype, PCR assays were performed to verify that the other genes were successfully integrated five different yeast colony - as described under *2.3 Rhodosporidium strain engineering* in the Materials section. The PCR products were analyzed with gel electrophoresis and the results are presented in *Figure 4* below. Two negative controls were used as PCR templates, BOT-A2 and H_2O, and one positive control (pDB31). *Figure 4* shows that all amplification targets were present in all yeast strains, which were stored as -80°C glycerol stocks and named TMB DB061-TMB DB073. In *Figure 4F* a lot of unspecific products are seen, which also can be seen for the negative control BOT-A2. PCR products from three randomly chosen strains, TMB DB061, TMB DB068 and TMB DB071, were sent for sequencing, confirming the correct sequences. Sequencing results were not obtained from the assay for amplifying the region from the PhaB terminator to the PhaC promotor assay due to the large amount of unspecific products.

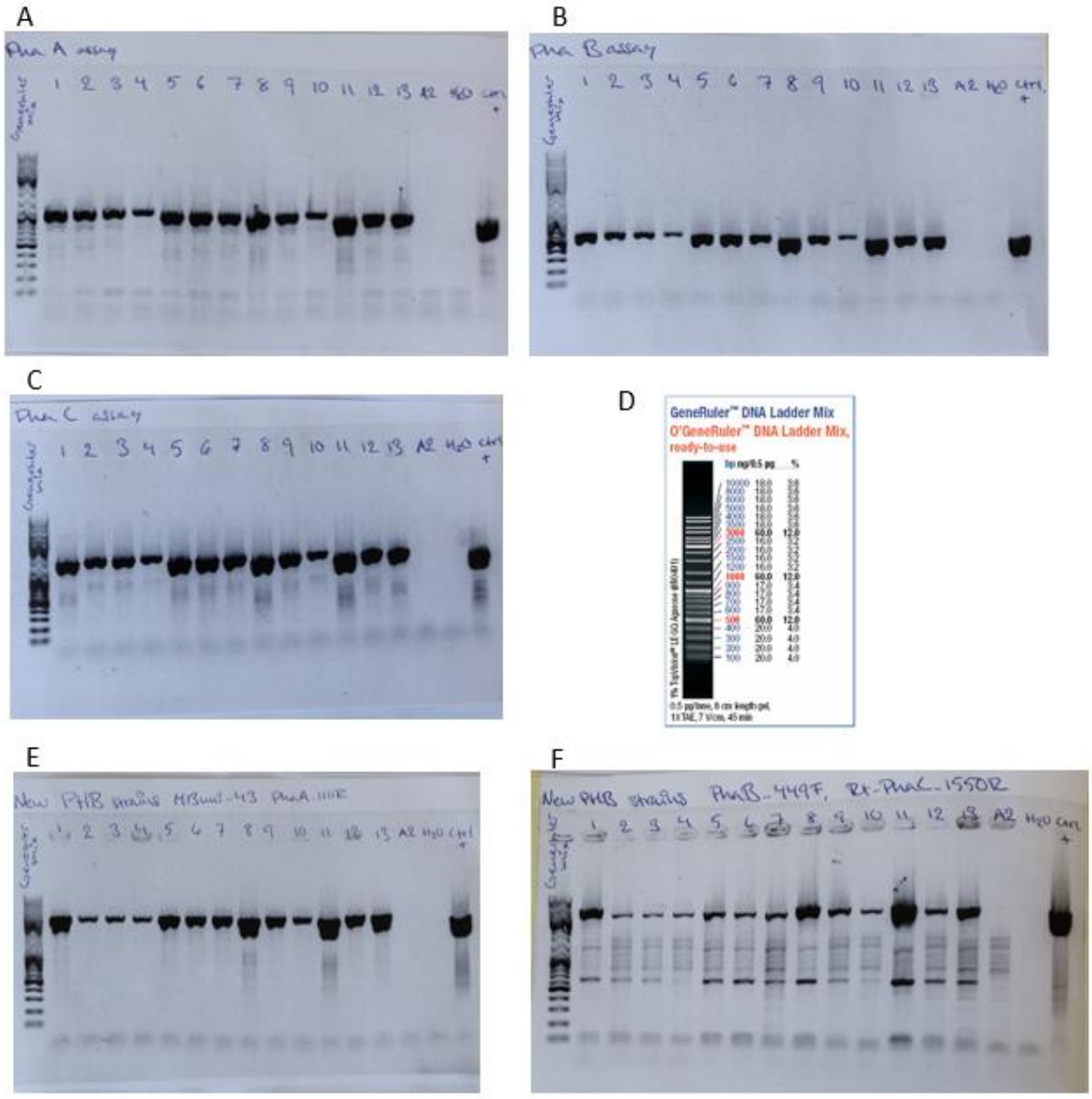

Figure 4: Gel pictures after yeast colony PCR on 13 transformants, negative controls (BOT-A2 and H_2O) and positive control (pDB31). A. PhaA assay. B. PhaB assay. C. PhaC assay. D. Generuler Mix ladder for size determination. E. PhaA promotor assay. F. PhaB terminator PhaC promotor assay.

3.2 Screening for PHB production

The 13 generated strains containing the inserted genes, BOT-A2 (wild type) and a control strain previously engineered to produce PHB, *S. cerevisiae* TMB4425 (de las Heras, et al., 2016), were cultivated for 72h in rich YPD medium with glucose for evaluating PHB production at the end of the cultivation. The screening results of PHB production are presented in *Figure 5* below. Under the used conditions *S. cerevisiae* TMB4425 produced 1.8 times more PHB than the engineered *R. toruloides* strain that produced most PHB, and only two of the generated strains (TMB DB066 and TMB DB067) produced PHB in measurable concentrations, i.e., more than the lower limit of detection in the standard curve (0.001g/l). Some of the other strains had visible chromatogram peaks at the correct retention time but were considered non-producers (0 g/L) since the values obtained were lower than the limit of detection.

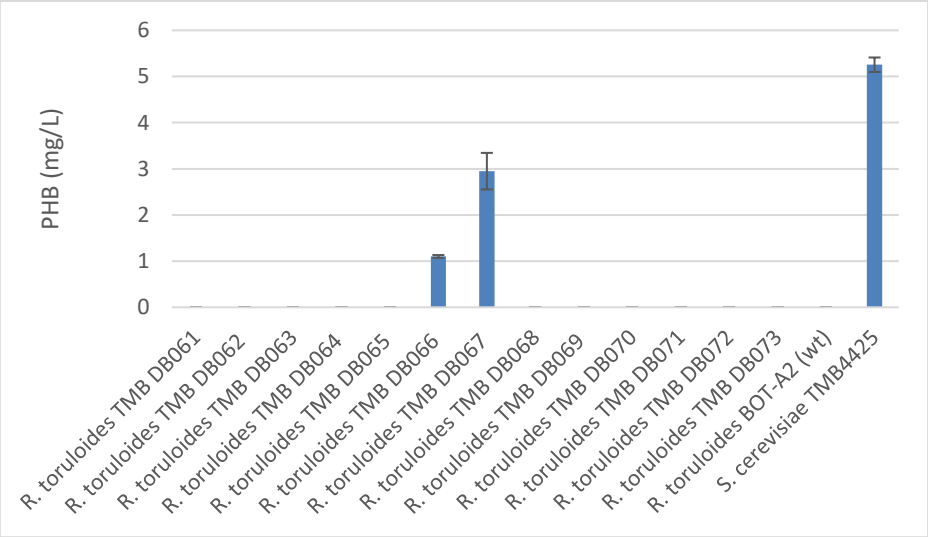

Figure 5: Screening for PHB production in transformed strains, BOT-A2 (wt) and TMB4425 (ctrl +) and observed production in two strains and the positive control. Error bars correspond to standard deviation of two biological replicates.

3.3 Evaluation of PHB producing strains

The two strains TMB DB066 and TMB DB067 proven to produce PHB in the screening were further cultivated, this time in defined minimal medium (YNB) with glucose to characterize and compare with wild type (BOT-A2, ctrl -) and a control strain (TMB4425, ctrl +). Evaluation of the PHB production capacity of the two constructed strains and the controls were performed in 1 L baffled shake flasks with 100 mL culture to enable more samples to be taken through the 72 h. Samples for OD_{620}, CDW, glucose, and PHB quantification were taken during the cultivations and are presented in *Figure 6* below. No bacterial contamination was found in any of the cultures according to microscopy analysis. Inclusion bodies were observed in the *R. toruloides* strains after 24 h and onwards which is likely due to lipid formation. Microscopy pictures where inclusion bodies can be seen in the *R. toruloides* strains at 24 h are presented in *8. Appendix* in *Figure A4-A7.*

It was expected that the two different organisms (*R. toruloides* and *S. cerevisiae)* would grow differently, which is seen in *Figure 6* looking at OD_{620} and CDW. A comparison of each measured parameter of the strains can be found in *Figures A1-A3* in *8. Appendix*. Both OD_{620} and CDW follows the same pattern compared to each other in all four cultures. The Crabtree-negative *R. toruloides* was able to grow to almost the double cell density compared to the Crabtree-positive *S. cerevisiae* under these conditions and *R. toruloides* reached its maximum biomass after about 24 h while *S. cerevisiae* continues to grow and first reached a plateau after about 48 h (likely diauxic growth, as ethanol production was detected from *S. cerevisiae (Figure 6D)*, which was not the case for *R. toruloides*). The two engineered strains TMB DB066 and TMB DB067 grew equally well as their parent strain BOT-A2 indicating that the integrated genes did not affect the growth of the cells. *S. cerevisiae* consumed glucose faster than the three *R. toruloides* strains. In the TMB4425 culture, glucose was depleted between 12 and 24 h, whereas in the three *R. toruloides* cultivations glucose was depleted between 24 and 26 h.

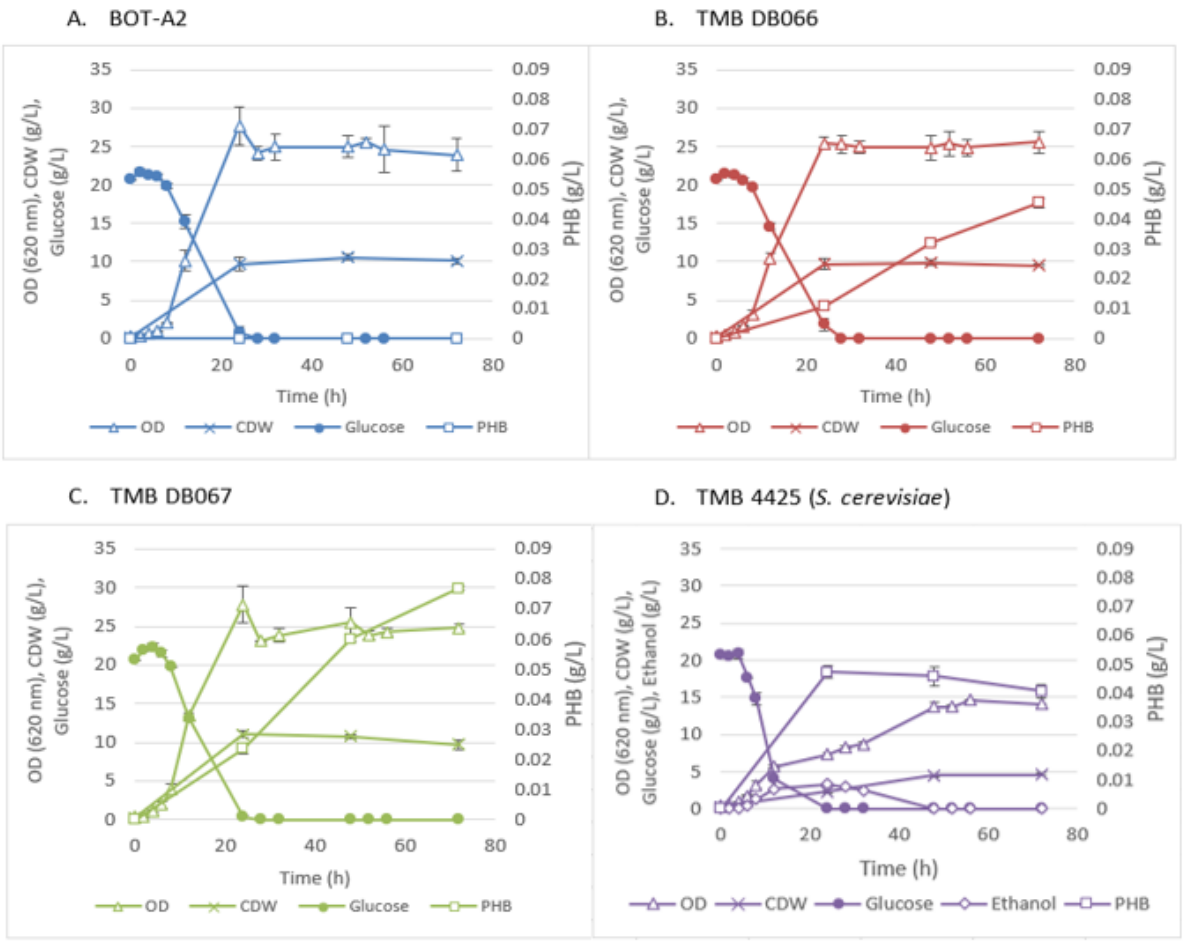

Figure 6: Aerobic growth and metabolite profiles from R. toruloides A. BOT-A2 (wt, ctrl -), B. TMB DB066, C. TMB DB067 and S. cerevisiae TMB4425 (ctrl +), with defined YNB medium and glucose in buffered shake flask cultivations. No ethanol was detected for R. toruloides. Error bars correspond to standard deviation of two biological replicates.

The production of PHB was followed once per day throughout the cultivations and is presented in *Figure 6* and directly compared between the strains in *Figure 7* below. As expected, the wild-type strain BOT-A2 did not produce any PHB. TMB4425 followed a pattern that have been observed before while grown on xylose, where the PHB level stabilizes after peaking (de las Heras, et al., 2016). In the current study, the peak for TMB4425 was observed at 24 h where the titer 0.047 g PHB/L was reached. TMB DB066 and TMB DB067, however followed a different pattern where increasing amounts of PHB was produced throughout the cultivations, without reaching a plateau. At 72 h TMB DB066 had reached a titer of 0.045 g PHB/L and TMB BD067 had reached 0.077 g PHB/L, long after the glucose was consumed. More process parameters are presented in *Table 4* below.

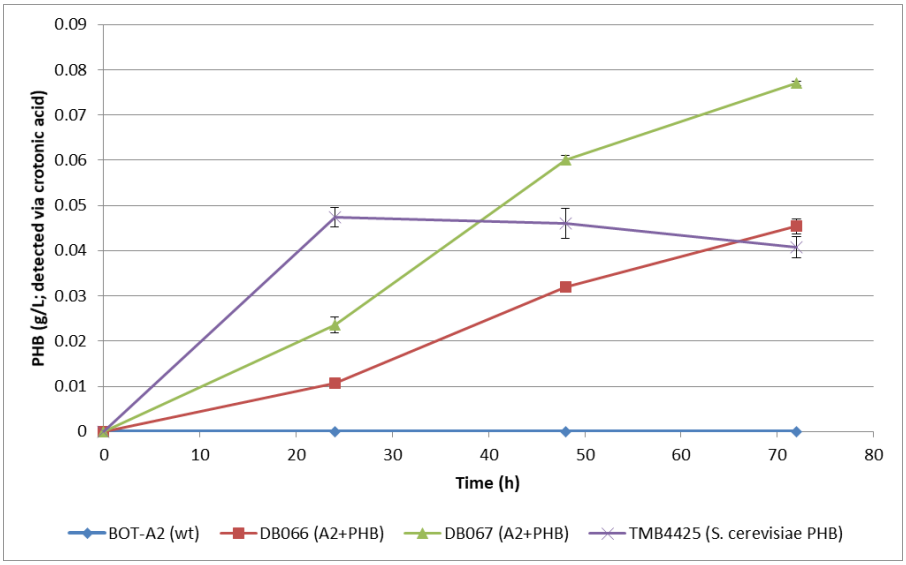

Figure 7: PHB production followed during shake flask cultivations for 72 h of R. toruloides BOT-A2 (wt, ctrl -), TMB DB066, TMB DB067 and S. cerevisiae TMB4425 (ctrl+). Error bars correspond to standard deviation of two biological replicates.

As seen in *Table 4* the two engineered *R. toruloides* strains produced more PHB/L than the *S. cerevisiae* strain TMB4425 under these specific conditions after 72 h. However, TMB4425 produced PHB more rapidly than the *R. toruloides* strains and reached its peak earlier. Therefore, *S. cerevisiae* reached its maximum yields and titers at an earlier time point than *R. toruloides.* When comparing the PHB yield in grams on carbon source (glucose) of *R. toruloides* TMB DB067 and *S. cerevisiae* TMB4425 after 72 h, more PHB per gram glucose was produced by *R. toruloides* TMB DB067. After 72 h *R. toruloides* TMB DB066 has reached a higher PHB yield on glucose in grams than *S. cerevisiae* TMB4425, but *R. toruloides* TMB DB066 never exceeded the PHB yield on glucose after 24 h of *S. cerevisiae* TMB4425. When the PHB yield on biomass is compared, *S. cerevisiae* TMB4425 produced more PHB per gram biomass than the two *R. toruloides* strains, as they formed more biomass.

Table 4: Process parameters for the strains R. toruloides BOT-A2 (wt, ctrl -), TMB DB066, TMB DB067 and S. cerevisiae TMB4425 (ctrl+) from two biological replicates are presented with standard deviation.

Titer: mg PHB /L	**0-24h**	**0-48h**	**0-72h**
R. toruloides BOT-A2	0 ± 0	0 ± 0	0 ± 0
R. toruloides TMB DB066	10.6 ± 0.2	32.0 ± 0.5	45.3 ± 1.7
R. toruloides TMB DB067	23.5 ± 1.8	60.1 ± 1.0	77.0 ± 0.5
S. cerevisiae TMB4425	47.4 ± 2.2	46.0 ± 3.3	40.8 ± 2.3
Yield: mg PHB /g glucose	**0-24h**	**0-48h**	**0-72h**
R. toruloides BOT-A2	0 ± 0	0 ± 0	0 ± 0
R. toruloides TMB DB066	0.565 ± 0.01	1.55 ± 0.02	2.19 ± 0.08
R. toruloides TMB DB067	1.16 ± 0.07	2.91 ± 0.05	3.72 ± 0.02
S. cerevisiae TMB4425	2.29 ± 0.1	2.23 ± 0.2	1.97 ± 0.1
Specific Yield: g PHB /g CDW	**0-24h**	**0-48h**	**0-72h**
R. toruloides BOT-A2	0 ± 0	0 ± 0	0 ± 0
R. toruloides TMB DB066	0.00112 ± 0.00005	0.00330 ± 0.0002	0.00489 ± 0.0003
R. toruloides TMB DB067	0.00218 ± 0.0002	0.00573 ± 0.0002	0.00816 ± 0.0006
S. cerevisiae TMB4425	0.0232 ± 0.003	0.0109 ± 0.001	0.00936 ± 0.0002

4. Discussion

In the present study it was shown that *R. toruloides* BOT-A2 can be genetically engineered with a large genetic construct (~14kb) consisting of several genes including promoters and terminators, by using electroporation. It was also demonstrated that *R. toruloides* codon-optimized genes from the PHB pathway from *C. necator* are functional in *R. toruloides* and that PHB can be produced in recombinant *R. toruloides*. The screening for PHB production in the 13 generated strains only resulted in two strains producing PHB in sufficient concentrations, although the yeast colony PCR showed that all genes were present in all the transformants. *R. toruloides* TMB DB068 was one of the strains where the PCR products of the colony PCR were sent for Sanger sequencing, which confirmed the correct sequences, but despite correct sequences, it did not produce any PHB. This could be explained by the fact that the electroporation method used relies on the non-homologous end-joining (NHEJ) repairing system, occurring in *R. toruloides* and resulting in random integration (Park, et al., 2018), instead of homologous recombination which is commonly used in e.g., *S. cerevisiae* for targeted integration. The random integration and the fact that the copy number integrated is unknown can explain why only two strains produced PHB. Previous results (Brink, unpublished) where a codon optimized gene of Green Fluorescent Protein (GFP) was integrated in *R. toruloides* showed a great difference between different clones, indicating that the integrated genes were expressed in different levels depending on the integration locus and copy number. As these results were known, it was expected that different levels of the enzymes would be expressed leading to different production of PHB. During the electroporation 1000 ng/kb of linearized plasmid DNA was given to the cells and it is possible that the genes are integrated in several copy numbers in different loci. The combination of unknown loci and

integrated copy number could explain why only two strains produced enough PHB for detection by HPLC, even though all strains are believed to have the genes integrated. Further experiments to determine integration loci and copy number through whole-genome sequencing would be interesting to understand the differences between the constructed strains. Another suggestion would be to perform an enzyme activity assay to determine the different levels of enzyme expressed in the different strains.

Before the linearized pDB31 was transformed into *R. toruloides* BOT-A2, another approach was tried where the gene construct consisting of PhaABC and KanMX with promoters and terminators (~12 kb) was amplified by PCR with Phusion polymerase (Thermo Scientific, USA). However, this large construct did not result in any PHB producing strains and the yeast colony PCR PhaA promotor assay did not result in any positive clones after the gel electrophoresis (data not shown). It has previously been shown that shorter fragments amplified by PCR and transformed into *R. toruloides* BOT-A2 result in functional recombinant strains and that shorter fragments result in higher transformation efficiency (data not shown). It is believed that this method did not work on the ~12 kb long fragment due to the length of the fragment. In the manual from the manufacturer, it is stated that Phusion polymerase (Thermo Scientific, USA) can amplify up to 7.5 kb from genomic DNA, so the ~12 kb plasmid DNA could be too long. Another possible reason could be the specificity of the primers used. If an incorrect fragment was amplified, the promoters, genes, terminators or parts of each would not be functional, resulting in clones not producing PHB.

The screening for PHB production, which was made using YPD medium, resulted in a PHB titer between 10 and 40 times lower than the cultivations where defined YNB medium was used, indicating that YPD is not optimal for screening for PHB production. It is possible that the remaining engineered *R. toruloides* strains would produce PHB in concentrations over the limit of detection if screening in defined YNB has been performed. The screening method used was designed to be simple: as previous studies on engineered *S. cerevisiae* had shown that it can take a couple of days to reach the final PHB concentrations in a culture (de las Heras, et al., 2016), cultures were incubated over the weekend (72h) and endpoint PHB was measured. This method did indeed identify strains that were producing more PHB, as was desired. However, with the much higher PHB titers found in YNB characterization, the screening method could be optimized in the future by the changing medium from YPD to YNB and adjusting the time the cells are cultured. As only endpoint samples were taken in the YPD screening, and the method used for PHB determination relies on the intracellular storage of PHB, it is possible that some PHB is missed if the cells start to lyse and release the PHB to the broth at the end of the cultivation. A shorter cultivation time could be preferable if an optimization is done in the future.

The final titer reached a total of 77 mg PHB/L for TMB DB067 cultured in defined YNB in shake flasks and TMB DB066 reached a titer of 45 mg PHB/L in total after 72 h of cultivation. The control strain *S. cerevisiae* TMB4425 reached a titer of 47 mg PHB/L after 24 h and 41 mg PHB/L after 72 h. Although *R. toruloides* TMB DB067 produced more PHB than the control strain under these conditions the comparison is not straightforward. The control strain *S. cerevisiae* TMB4425 was selected as the best PHB producing strain available at the Department of Applied Microbiology (TMB). *S. cerevisiae* TMB4425 has previously been reported to produce up to 730 mg PHB/L (Portugal-Nunes, et al., 2017) while grown anaerobically on xylose and under optimized conditions for the specific strain, corresponding to a 10 times higher titer than *R. toruloides* TMB DB067 in this study. Another difference is the genes used when engineering the organisms. There are two different acetoacetyl-CoA reductases (encoded by different variants of PhaB) using different cofactors, one NADPH-dependent and one NADH-dependent (Taguchi, et al., 2012). *S. cerevisiae* TMB4425 was engineered with the NADH-dependent acetoacetyl-CoA reductase, making it more suitable for anaerobic conditions, while in this

study *R. toruloides* TMB DB066 and TMB DB067 was engineered with the NADPH-dependent enzyme. The strategy used in this study is based on Sandström, et al., 2015 where *S. cerevisiae* was engineered and the strain TMB4443 (NADPH-dependent PhaB) was made to produce PHB from xylose with the NADPH-dependent enzyme. In their study the final titer reached a total of 45 mg PHB/L for TMB4443 in shake flask conditions and increased to 102 mg PHB/L in well-aerated bioreactor (Sandström, et al., 2015). If the shake flask condition is compared with *R. toruloides* TMB DB067 in this study 1.7 times more PHB is produced. For future experiments to make the comparison easier, *S. cerevisiae* TMB4443 can be used as a control strain, cultivated under the same conditions used in this study and in future experiments. To further evaluate TMB DB067, cultivations in a bioreactor could be performed as other studies have reached higher titers in bioreactors compared to shake flasks (de las Heras, et al., 2016 and Sandström, et al., 2015). When comparing the results from this study with previous results in bacteria (*C. necator* DSM545 (Mozumder, et al., 2014) and recombinant *E. coli* GCSC 6576 (Kim, 2000)) the cultivation conditions are very different from this study, using different substrates, bioreactors using a fed batch setup for of the carbon source, nitrogen limitation (Mozumder, et al., 2014) and oxygen limitation (Kim, 2000). The very different conditions used in these different studies make a direct comparison difficult, and further characterization of *R. toruloides* TMB DB067 would be needed for more comparable results.

If the specific yield on biomass in this study is compared between *S. cerevisiae* and the engineered *R. toruloides* strains, there is a big difference especially in the beginning of the cultivation due to the different growth profiles (*Table 4*). After 24 h *S. cerevisiae* has reached the maximum titer of PHB but not reached a high cell density, whereas *R. toruloides* has reached maximum cell density but only around a third of the PHB produced during the whole cultivation. When the cells are cultivated for 72 h the difference between *S. cerevisiae* and *R. toruloides* has decreased as *R. toruloides* continues to produce PHB, while maximum cell density is reached. Comparing the PHB yield in grams of carbon source (glucose) of *R. toruloides* TMB DB067 and *S. cerevisiae* TMB4425 after 72 h, more PHB per gram glucose is produced by *R. toruloides* TMB DB067 than *S. cerevisiae* TMB4425. This could be explained by *S. cerevisiae* producing other products where the carbon is used, e.g., ethanol, acetate and glycerol. These metabolites have been measured in other studies where *S. cerevisiae* have been used as a production host (Sandström, et al., 2015), but were not quantified in this study due to time limitations and the fact that the focus was on *R. toruloides*. Ethanol production was however quantified from the culture broth, and it can be seen in *Figure 6D* that that it is formed in the beginning of the culturing and then decreases again after the glucose is depleted. Moreover, in the culture broth taken for glucose samples, no extracellular metabolites were detected in the *R. toruloides* samples, indicating that no extracellular byproducts were formed where carbon can be lost. Although no byproduct formation is good for *R. toruloides* as a production host, more samples and analysis are required to make sure that no extracellular byproducts are formed.

The PHB production in the engineered *R. toruloides* strains continued throughout the cultivations even after glucose depletion, which was not expected as glycolytic promotors were used, and the precursor acetyl-CoA is synthesized in the cytosol under nutrient limited conditions but in the presence of glucose as carbon source (Fakas, 2017 and Park, et al., 2018). Since inclusion bodies, believed to be a sign of lipid production, were observed in the microscope already at 24 h, it is possible that any lipids formed early in the cultivation were converted back to another intermediate for use as maintenance energy in the later stages of the cultivations after the glucose had been fully consumed. If some of the proposed “maintenance” carbon, e.g., in the form of acetyl-CoA, could be catalyzed into acetoacetyl-CoA and further into PHB, it could explain the increase in PHB that continued even after glucose depletion. No lipid analysis was performed in this study, but future investigation of lipid production during the cultivation of the engineered *R. toruloides* could provide an answer if the lipids are

consumed by the cells after the carbon source is depleted. It would also be interesting to see for how long the PHB keeps increasing during a cultivation, since the PHB concentration had not plateaued after 72 h in the YNB cultivations (*Figure 6*).

Another unexpected result from the cultivations was that the two strains TMB DB066 and TMB DB067 grew equally well as their parent strain BOT-A2, indicating that the integrated genes did not affect the fitness of the cells. Acetyl-CoA is required for cell growth in the mitochondria while PHB is synthesized from cytosolic acetyl-CoA. However, it was expected that the usage of acetyl-CoA for PHB production would affect the cell growth, as oleaginous yeast has an efflux of mitochondrial citrate into the cytosol where it is cleaved into acetyl-CoA and oxaloacetate (Vorapreeda et al., 2011). Acetyl-CoA needed for fatty acid synthesis comes from the glycolytic pathway where acetate is converted into acetyl-CoA in the cytosol (Fakas, 2017 and Park, et al., 2018). Since lipids are believed to be formed in parallel to the PHB production in the engineered strains, the cytosolic acetyl-CoA would then be used for both lipid formation and PHB synthesis. The citrate efflux from the mitochondria together with the cytosolic acetyl-CoA formed from the glycolytic pathway used for PHB production was expected to affect growth because of the reduced availability of mitochondrial acetyl-CoA, but this was not observed during the physiological characterization of the recombinant strains.

With these results proving that PHB can be produced in recombinant *R. toruloides* BOT-A2 future engineering can be done for improvement. In natural producers, PHB is produced, under nutrient limitation with the carbon source in excess, as an intracellular carbon storage (Kocharin, et al., 2012). Lipid production in *R. toruloides* serves the same function as a carbon and energy storage during nutrient limitations (often nitrogen) (Park, et al., 2018). The next engineering step could be to exchange the constitutive glycolytic promoters used in this study to promoters that are induced under nitrogen starvation to evaluate if more PHB can be produced. As lipids are synthesized under nitrogen starvation it is likely that lipids and PHB are produced in parallel competing for the acetyl-CoA as a common precursor. To avoid this and improve PHB titer and yields, metabolic engineering to delete genes responsible for lipid production could be tried. As *R. toruloides* can produce lipids up to 65% of the CDW (Park, et al., 2018), substituting the native lipid production pathways with the PHB genes is a promising strategy that could possibly increase the PHB titers in recombinant *R. toruloides*.

5. Conclusion

This study demonstrates that PHB production in recombinant *R. toruloides* BOT-A2 is possible and two PHB producing strains were constructed. This project is in an early stage and further optimization is needed for obtaining higher titer and yields and more experiments are needed to understand the PHB production process in the engineered *R. toruloides*. However, the current results are encouraging as they demonstrate that *R. toruloides* is a promising host for PHB production.

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8. Appendix

Abbreviations

Acetyl-CoA - Acetyl coenzyme A

CDW – Cell dry weight

Ctrl+ - Positive control

Ctrl- - Negative control

DS - Downstream

GFP – Green fluorescent protein

HPLC – High Performance Liquid Chromatography

NADH - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

NHEJ – Non-homologous end-joining

OD – Optical density

PCR - Polymerase chain reaction

PHA - Polyhudroxyalcanoates

PHB – Polyhydroxybuturate

TAG – Triacylglycerol

US - Upstream

Wt – Wild-type

Results

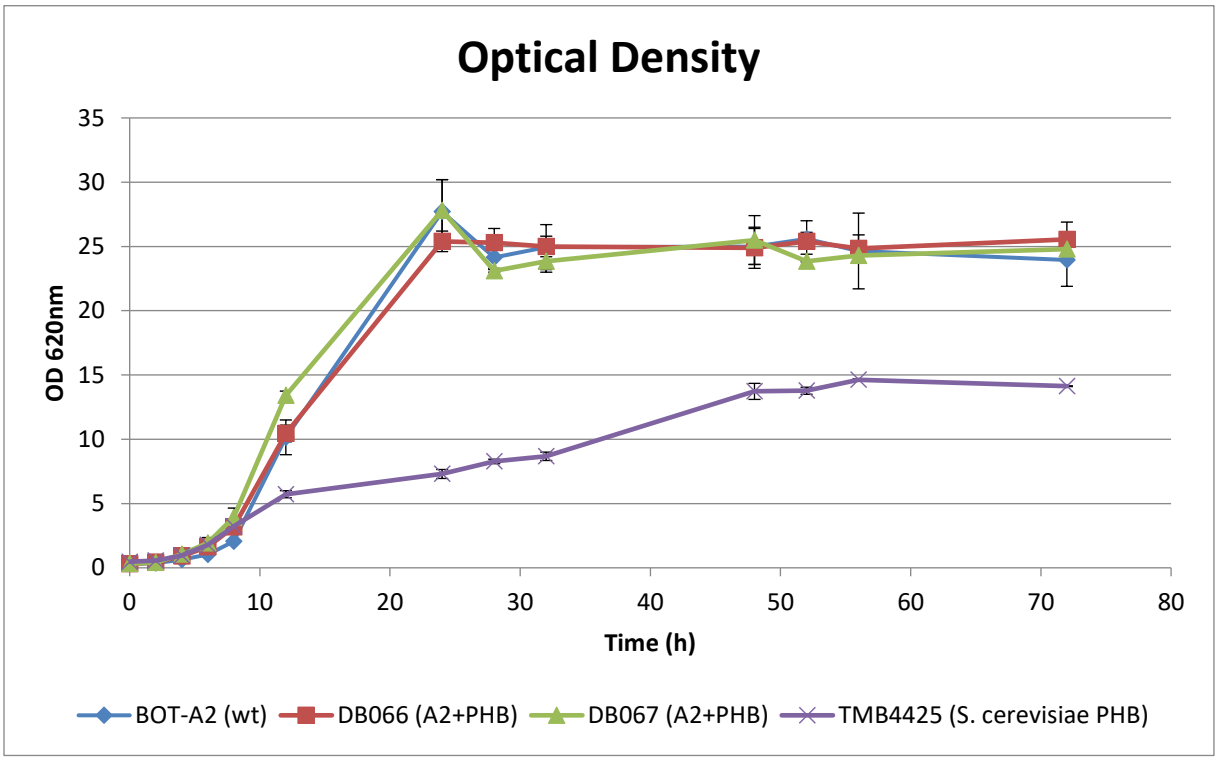

Figure A1: Optical density followed during shake flask cultivations for 72 h of R. toruloides BOT-A2 (wt, ctrl -), TMB DB066, TMB DB067 and S. cerevisiae TMB4425 (ctrl+). Error bars correspond to standard deviation of two biological replicates.

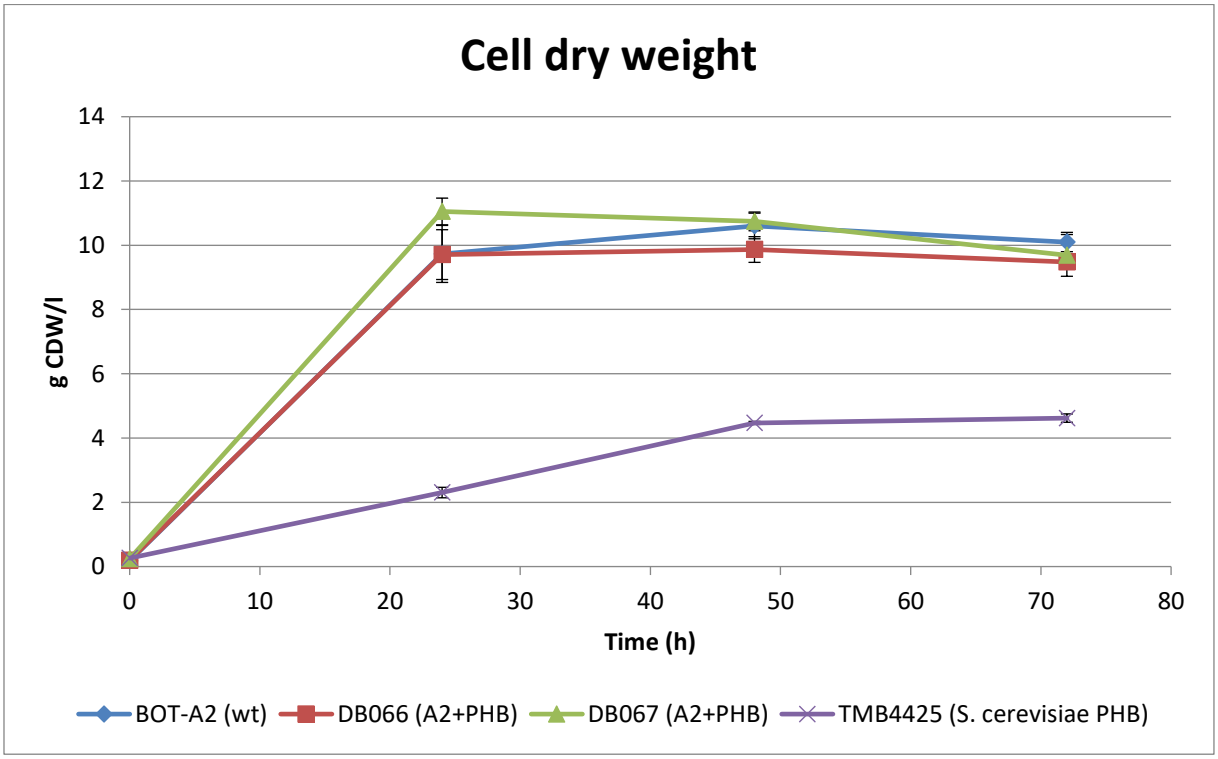

Figure A2: Cell dry weight followed during shake flask cultivations for 72 h of R. toruloides BOT-A2 (wt, ctrl -), TMB DB066, TMB DB067 and S. cerevisiae TMB4425 (ctrl+). Error bars correspond to standard deviation of two biological replicates.

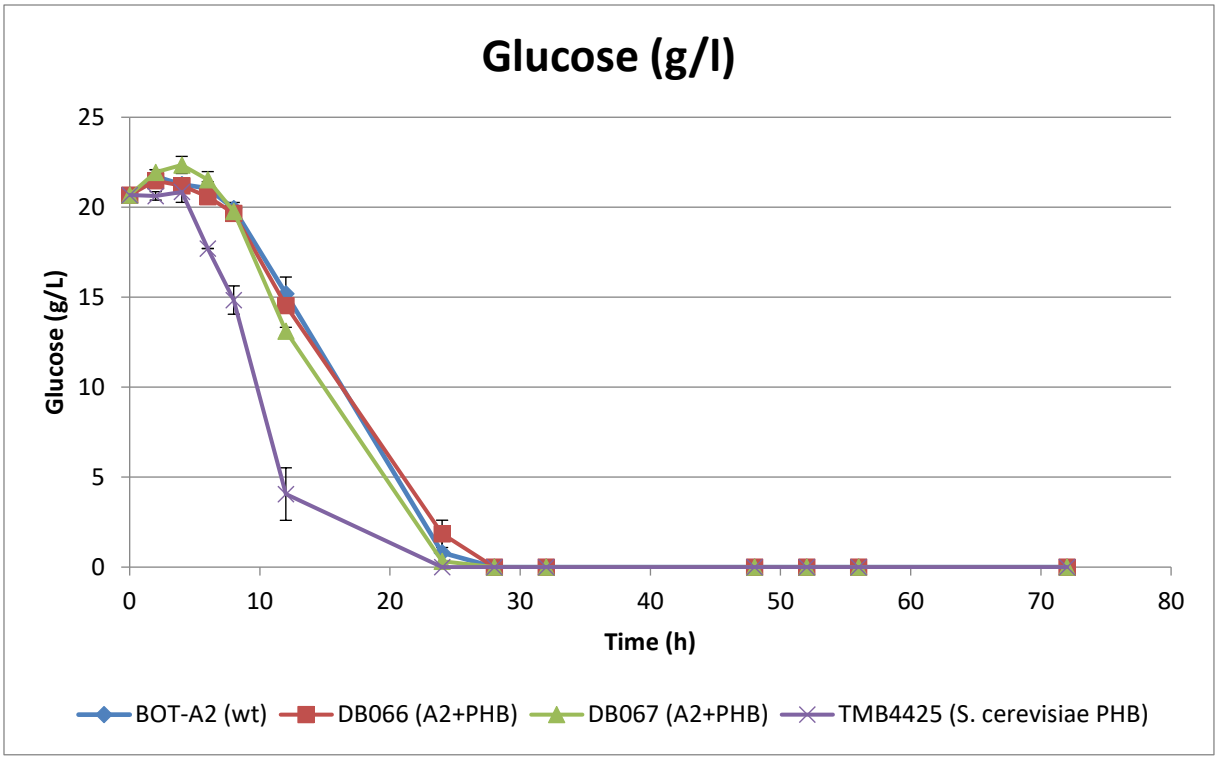

Figure A3: Glucose consumption followed during shake flask cultivations for 72 h of R. toruloides BOT-A2 (wt, ctrl -), TMB DB066, TMB DB067 and S. cerevisiae TMB4425 (ctrl+). Error bars correspond to standard deviation of two biological replicates.

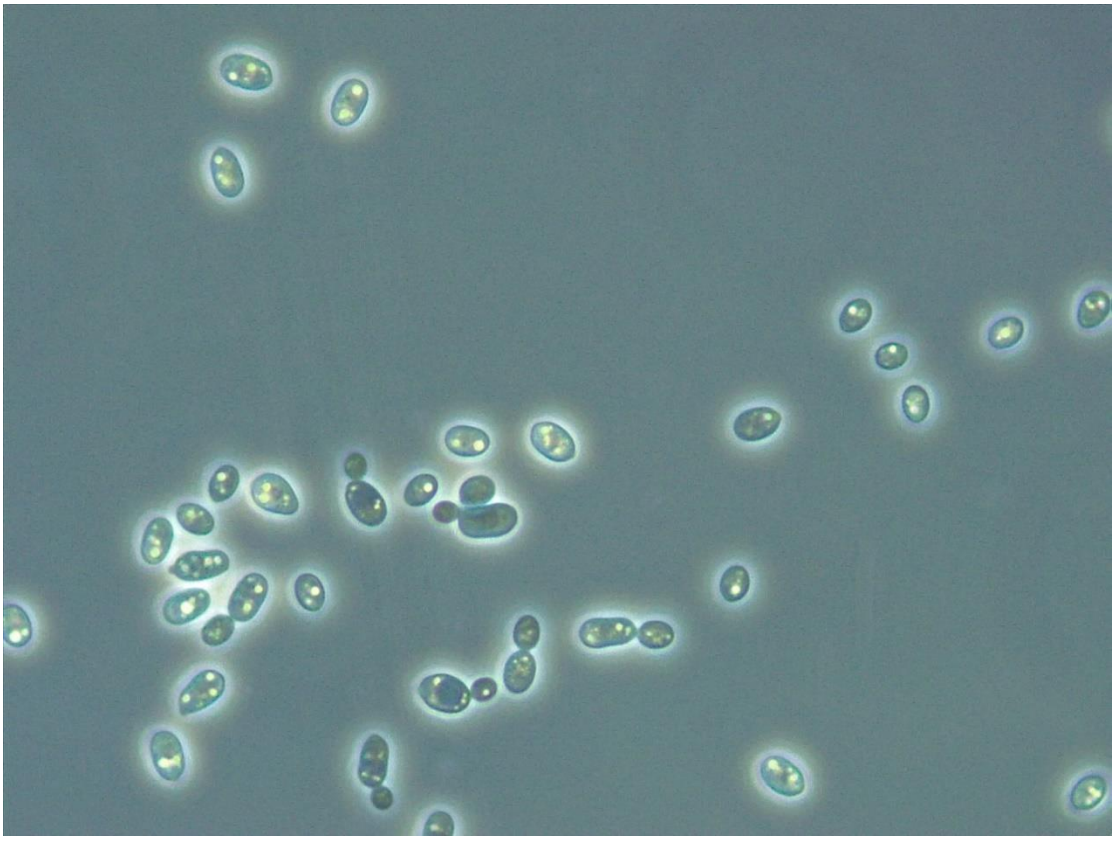

Figure A4: Microscopy sample (100x) of R. toruloides BOT-A2 (wt) after 24 h, diluted 10 times, where inclusion bodies can be seen inside the cells.

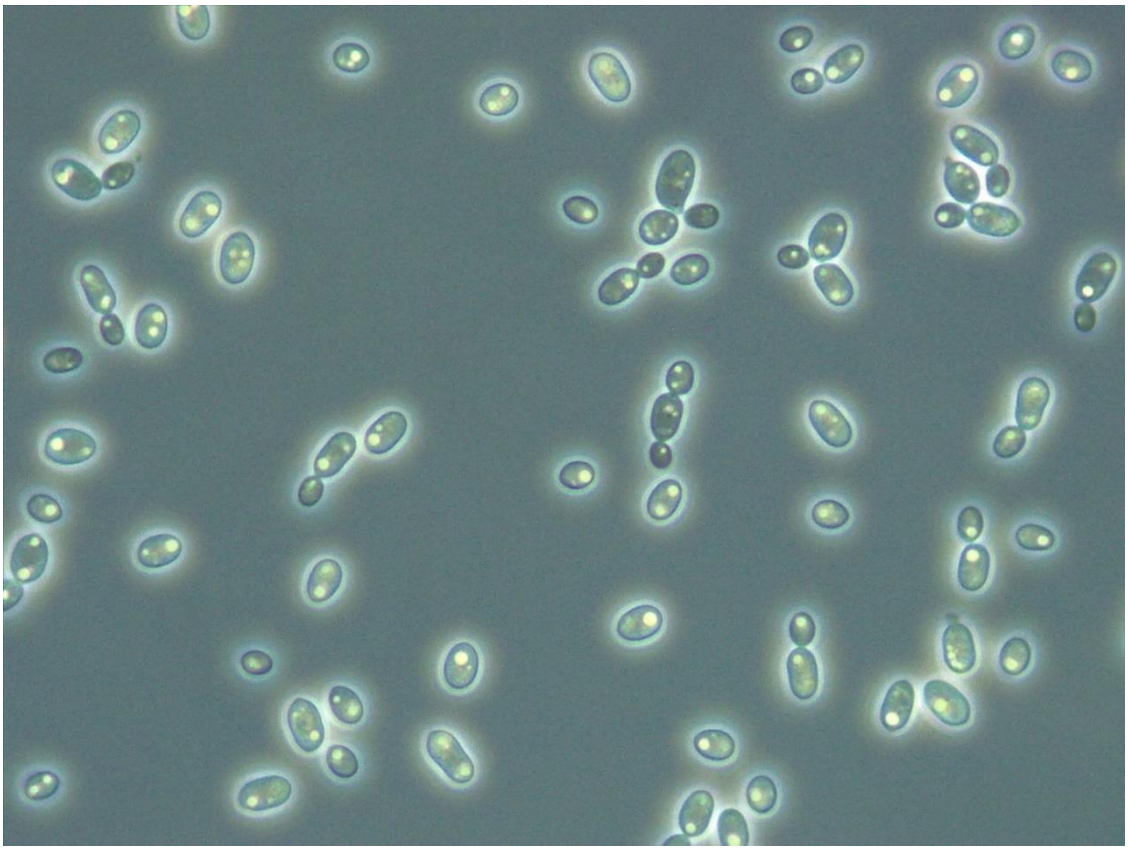

Figure A5: Microscopy sample (100x) of R. toruloides TMB DB066 after 24 h, diluted 10 times, where inclusion bodies can be seen inside the cells.

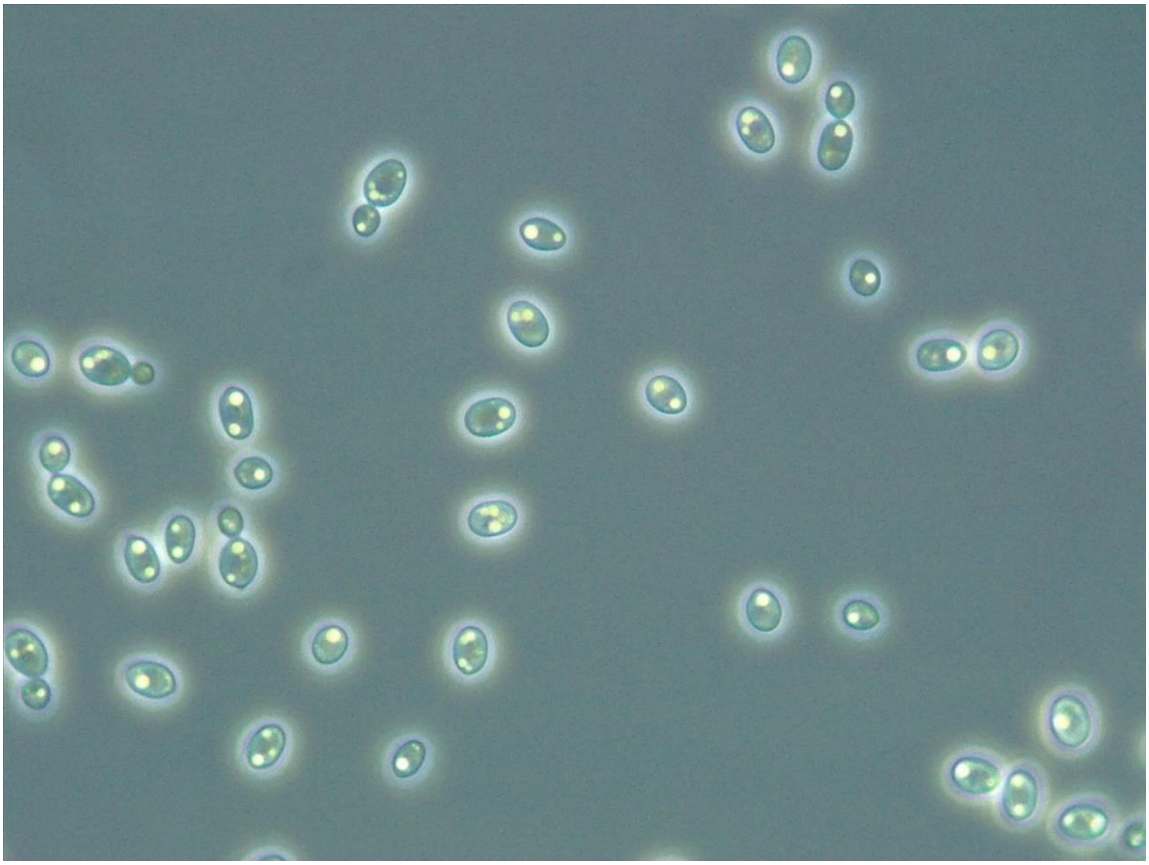

Figure A6: Microscopy sample (100x) of R. toruloides TMB DB067 after 24 h, diluted 10 times, where inclusion bodies can be seen inside the cells.

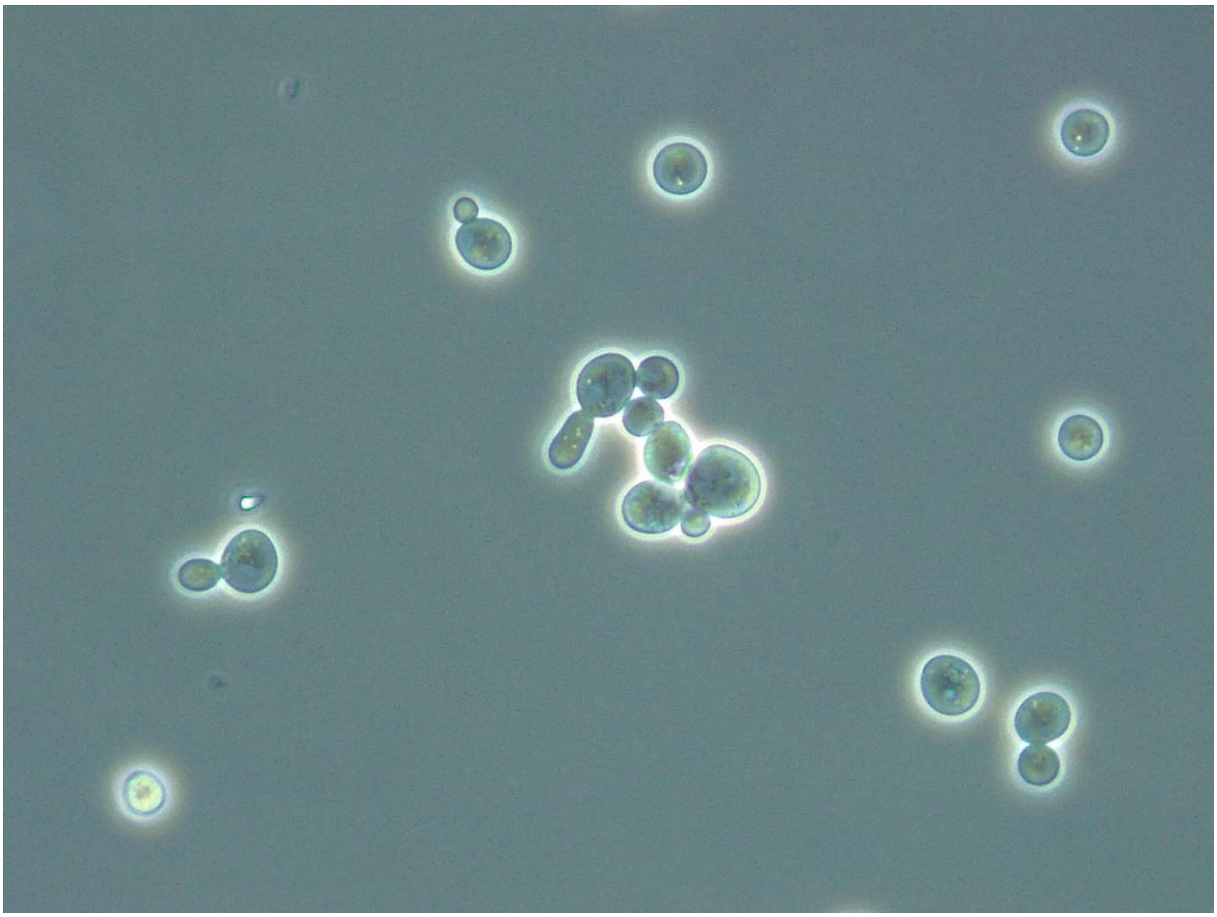

Figure A7: Microscopy sample (100x) of S. cerevisiae TMB4425 after 24 h, diluted 10 times, no inclusion bodies seen inside the cells.