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Massachusetts Institute of Technology

**Original paper**

**Title:**

# Engineering *Yarrowia lipolytica* for poly-3-hydroxybutyrate production

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**Abstract**

Strains of *Yarrowia lipolytica* were engineered to express the poly-3-hydroxybutyrate (PHB) biosynthetic pathway. The genes for β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHB synthase were cloned and inserted into the chromosome of *Y. lipolytica*. In shake flasks, the engineered strain accumulated PHB to 1.50 % and 3.84 % of cell dry weight in complex medium supplemented with glucose and acetate as carbon source, respectively. In fed-batch fermentation using acetate as sole carbon source, 7.35 g/l PHB (10.2 % of cell dry weight) was produced. Selection of *Y. lipolytica* as host for PHB synthesis was motivated by the fact that this organism is a good lipids producer, which suggests robust acetyl-CoA supply also the precursor of the PHB pathway. Acetic acid could be supplied by gas fermentation, anaerobic digestion and other low cost supply route.

*Keywords*: Poly-3-hydroxybutyrate, PHB, Metabolic Engineering, *phaCAB*, Acetate, *Yarrowia lipolytica*

1 **1. Introduction**

2 Microbial production of biodegradable polymers from renewable feedstock draws
3 increasing attention due to the growing concerns about the negative environmental impact of
4 petroleum-derived plastics and depletion of fossil fuels (Philip et al. 2007).
5 Polyhydroxyalkanoates (PHAs) are a family of the most promising bio-based and
6 biodegradable polyesters that possess diverse material properties. A series of recombinant
7 hosts have been constructed to express PHA biosynthetic genes to explore their potential as
8 bio-based plastic producers (Suriyamongkol et al. 2007).

9 Acetyl-CoA is a central metabolite in carbon and energy metabolism which connects
10 glycolysis, tricarboxylic acid cycle, β-oxidation and *de novo* biosynthesis of fatty acids.
11 Starting from acetyl-CoA, the biosynthesis of poly-3-hydroxybutyrate (PHB), the simplest
12 and most well-studied member of PHA family, is catalyzed by three enzymes: β-ketothiolase,
13 NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase (Suriyamongkol et al.
14 2007). Intracellular availability of acetyl-CoA enables PHB accumulation in a variety of
15 non-natural producers including *Escherichia coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*,
16 *Arabidopsis thaliana*, cotton, maize, tobacco, and insect culture cells (Carlson and Srienc
17 2006).

18 PHA are produced as intracellular inclusion bodies, thus the cell size is regarded as an
19 important factor limiting the amount of PHA granules and the total quantity of PHA that can
20 be accumulated in each cell (Jiang et al. 2015). Generally, most bacteria cells have the size
21 ranging from 0.5 to 2 μm. The manipulation of cell morphology related genes was reported to
22 enlarge bacterial shapes and improve PHB accumulation in *E. coli*, and PHB could reach
23 more than 80 % of cell dry weight (CDW) (Wu et al. 2016a; Wu et al. 2016b). In terms of cell

size, yeast cells are much bigger than bacteria cells, which might help to store more inclusion bodies. *S. cerevisiae*, one of the most attractive cell factory platform for industrial production of fuels and chemicals, has been well studied to evaluate its potential for PHB production. When PHA synthase was expressed, recombinant *S. cerevisiae* was able to accumulate 0.5 % PHB of CDW in bioreactor cultivations (Leaf et al. 1996). Co-expression of β-ketothiolase and acetoacetyl-CoA reductase was found to improve PHB content to 7.5 % of CDW (Carlson and Srienc 2006). Overexpression of the ethanol degradation pathway (Kocharin et al. 2012) and phosphoketolase pathway (Kocharin et al. 2013) were also demonstrated to increase the cytosolic acetyl-CoA pools and boost PHB production. However, the best performance of PHB titer achieved in *S. cerevisiae* was below 0.2 mg/l in shake flask cultivations (Kocharin et al. 2013). Acetyl-CoA serves as the precursor for PHB biosynthesis and efficient supply of acetyl-CoA is crucial for increasing PHB production (Carlson and Srienc 2006). The carbon metabolic flux of *S. cerevisiae* on glucose or xylose may be naturally regulated to favor ethanol production, which limits its metabolic potential for efficient PHB accumulation (Kocharin et al. 2013; Sandstrom et al. 2015).

*Yarrowia lipolytica* is an obligate aerobic, oleaginous yeast which can grow on broad substrates including carbohydrates, alkanes, fatty acids, and triglycerides. It is categorized as GRAS (generally recognized as safe) host and has been engineered for the commercial production of omega-3 eicosapentaenoic acid (EPA) (Xue et al. 2013). The engineered *Y. lipolytica* was recently proved to be a superior lipid cell factory with high yield (84.7% of theoretical yield), titer (55 g/l), and productivity (1 g/l/h within the stationary phase) (Qiao et al. 2015). Both lipid biosynthesis and PHB production are highly dependent on intracellular availability of acetyl-CoA and reducing equivalent NADPH. This is the basis for investigating

1 *Y. lipolytica* for biopolymer production. In this study, the PHB synthetic pathway was built in

2 *Y. lipolytica*, and the engineered strain exhibited promising PHB producing ability when

3 acetate was employed as carbon source.

4

5 **2. Materials and methods**

6 *2.1 Bacterial strains, media, and culture conditions*

7 The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* NEB-5α

8 was cultivated in Luria-Bertani (LB) medium (5 g/l yeast extract, 10 g/l Bacto tryptone, and

9 10 g/l NaCl) at 37°C and used as host strain for plasmids construction. *Y. lipolytica* Po1g was

10 purchased from Yeastern Biotech Corporation (Taiwan, China) and cultivated in YPD media

11 (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) at 30°C.

12 *2.2 Plasmid construction*

13 Standard procedures or manufacturers' instructions were followed for plasmid

14 construction. Oligonucleotides were synthesized by Sigma-Aldrich (St. Louis, MO) and are

15 listed in Table 2. PHB biosynthetic genes *phaA*, *phaB*, and *phaC* were cloned from the

16 genomic DNA of *Ralstonia eutropha*. The type II PHA synthase gene *phaC1* from

17 *Pseudomonas sp*. 61-3 with Ser325Thr/Gln481Lys mutations was custom-synthesized by Life

18 Technologies (Grand Island, NY).

19 To construct pQK1, the GPD promoter (1 kb region upstream to *Y. lipolytica*

20 glyceraldehyde-3-phosphate dehydrogenase gene) and POX1 terminator (500 bp region

21 downstream to *Y. lipolytica* lipase 1 gene) were amplified and combined in pUC19 vector. The

22 same cloning strategy was applied to make pQK3, featuring EXP1 promoter and POX1

1 terminator. PHB biosynthetic genes *phaA* and *phaB* were respectively introduced into pQK1
2 and pQK3 to generate pQK1-phaA and pQK3-phaB. The pMT15-phaC and pMT15-phaC1
3 were respectively constructed by inserting *phaC* and *phaC1* into pMT15 vector. The entire
4 PHB synthetic operons were constructed *via* assembling the transcription units
5 (promoter-gene-terminator) from pQK1-phaA, pQK3-phaB and pMT15-phaC or
6 pMT15-phaC. Gibson assembly was exclusively applied to link DNA fragments.

7 *2.3 Yeast transformation*

8 *Y. lipolytica* Po1g was transformed with the linearized plasmid pMT15-CAB (*Ase*I
9 digestion) or pMT15-C1AB (*Not*I digestion) according to the protocol reported previously
10 (Qiao et al. 2015), which allowed for the chromosomal integration of PHB biosynthetic
11 operon and generated *Y. lipolytica* CAB and *Y. lipolytica* C1AB, respectively. Transformants
12 were selected on defined medium (6.7 g/l yeast nitrogen base without amino acids
13 supplemented with, 0.67 g/l CSM-leucine, 20 g/l glucose and 16 g/l agar). The successful
14 engineered colonies were validated by PCR amplification of the integrated gene, using
15 extracted genomic DNA as template.

16 *2.4 PHB production in shake flask and bioreactor cultures*

17 For PHB production in shake flasks, the engineered *Y. lipolytica* strain was cultivated at
18 30°C for 24 h and then inoculated into 250 ml conical flasks containing 25 ml cultivation
19 medium at an inoculation volume of 1%. Three different kinds of media were employed,
20 including YNB (6.7 g/l yeast nitrogen base without amino acids and 20 g/l glucose), YPD50
21 (10 g/l yeast extract, 20 g/l peptone and 50 g/l glucose), and YPA (10 g/l yeast extract, 20 g/l
22 peptone and 20 g/l sodium acetate).

1 For PHB production in bioreactors, seed culture of *Y. lipolytica* CAB was inoculated into
2 a 3-l fermentor (Bioflo 115, Eppendorf) at 1% inoculation volume with an operating volume
3 of 1.5 l. The starting cultivation medium was defined medium (13.4 g/l yeast nitrogen base
4 without amino acids) or complex medium (10 g/l yeast extract and 20 g/l peptone)
5 supplemented with 50 g/l sodium acetate as carbon source. Oxygen was provided by sparging
6 filtered air at a flow rate of 2 l $min^{-1}$ and maintained at 20% of air saturation by adjusting the
7 agitation rate in the 200-800 rpm range. The temperature was maintained at 28°C and the pH
8 and concentration of acetate in the bioreactor were maintained at 6.5 and ~27 g/L by
9 automatic addition of 500 g/l acetic acid solution.

10 *2.5 Analytical methods*

11 *Y. lipolytica* cells were collected by centrifugation at 8,000 g for 10 min. Cell pellets
12 were washed with distilled water and lyophilized for cell dry weight (CDW) measurement.
13 For intracellular PHB content analysis, the polymer was degraded and converted to
14 methy-3-hydroxybutyrate by methanolysis at 100°C for 4 h in the presence of 3% (v/v) $H_2SO_4$
15 and then quantified by gas chromatograph (GC). PHB purchased from Sigma-Aldrich was
16 used as standard.

17 Intracellular PHB polymers were isolated from the lyophilized *Y. lipolytica* cells with
18 chloroform in screw-capped tubes at 100°C for 4 h. The chloroform solution of PHB was
19 collected by centrifugation and subsequently precipitated in an excess of 10 volumes of
20 ice-cold n-hexane. For the molecular weight assay of PHB, the extracted samples were
21 applied to analytical gel permeation chromatography (GPC) (LC-20AD, Shimadzu, Japan)
22 equipped with Shodex K-804 column (Waters, USA). Polystyrene standards purchased from
23 Sigma-Aldrich were used for calibration.

1 *2.6 Total RNA isolation and quantitative PCR analysis*

2 The total RNA (approximately 100 µg) was isolated from *Y. lipolytica* cells obtained
3 from shaking flask cultures at proper culturing time (36 h) using the RiboPure-Yeast kit
4 (Thermo Fisher Scientific). The RNA was digested with DNase I to remove the residual
5 DNAs. RNA was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop
6 Technologies, Wilmington, DE) and samples were stored at -80°C until the qPCR analysis.
7 0.5 µg of DNase-treated RNA samples were used to make cDNA using ImProm-II™ Reverse
8 Transcription kit (Promega) and random hexamer for 60 min at 42°C according to the
9 manufacturer's instruction.

10 The cDNA levels were quantified using a Biorad iCycler 4 Real-Time PCR Detection
11 System (Bio-Rad) with SYBR Green I detection. Each sample was prepared in triplicate in a
12 96-well plate (VWR) and the reaction mixture (30 µL final volume) contains 1×
13 XtensaMix-SG (BioWORKS), 200 nM primer, 2.5 mM $MgCl_2$, and 0.75 U of Taq DNA
14 polymerase (New England Biolabs). Real time PCR was performed with an initial
15 denaturation of 3 min at 95°C, followed by 30 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at
16 72°C. The threshold cycles (Ct) were calculated using the iCycler software. Primer dimers in
17 all the assays showed distinct melt characteristics from the desired amplicons. The real-time
18 PCR primers used in this study were presented in Supplementary Table 1.

19

20 **3. Results and discussion**

21 *3.1 Construction of integrative PHB expression vectors*

22 Wild type oleaginous yeast *Y. lipolytica* is not capable of producing PHB. To impart the

1 ability of PHB accumulation in *Y. lipolytica*, three heterologous enzymes, namely
2 β-ketothiolase (encoded by *phaA*), acetoacetyl-CoA reductase (encoded by *phaB*), and PHA
3 synthase (encoded by *phaC*) need to be expressed. To this end, the PHB biosynthetic operon
4 of *R. eutropha* H16 was chosen due to its demonstrated effectiveness in enabling PHB
5 production in *S. cerevisiae* previously. Moreover, the representative Class II PHA synthase,
6 Ser325Thr/Gln481Lys mutant of PHA synthase from *Pseudomonas sp*. 61-3 (encoded by
7 *phaC1*), which possesses broad substrate specificity ranging from C3-C12 carbon atoms
8 (Matsusaki et al. 2000; Taguchi et al. 2008), was also chosen to be expressed in *Y. lipolytica*.

9 All genes were cloned with a strong constitutive promoter (pGPD, pEXP1, and pTEF for
10 *phaA*, *phaB* and *phaC*/*phaC1*, respectively) into a single integrative vector, named
11 pMT15-CAB or pMT15-C1AB (Fig. 2). All plasmids were sequenced and validated.

12 *3.2 Strain engineering*

13 *Y. lipolytica* strain Po1g was used as the parent host for PHB synthesis. Seven colonies
14 were selected for pMT15-CAB and pMT15-C1AB transformation, respectively, followed by
15 72 h cultivation in 50 ml Falcon tubes containing 5 ml YPD medium to evaluate their PHB
16 production capacity (Fig. 3 A).

17 All strains were capable of accumulating intracellular PHB biopolymers, and the PHB
18 content with *phaC* from *R. eutropha* was much higher than that obtained with *phaC1* from
19 *Pseudomonas sp.* 61-3. Subsequently, quantitative real-time PCR of PHA synthase genes were
20 performed to study the transcriptional level of *phaC1* and *phaC* in different *Y. lipolytica*
21 strains (Supplementary Fig. 1). As shown, the transcriptional level of *phaC* was significantly
22 higher than that of *phaC1*, which might lead to the phenomenon of improved PHB production.
23 Consequently, *Y. lipolytica* CAB was selected for the following PHB producing experiments.

1 *3.3 PHB production in shake flask cultures*

2 The engineered *Y. lipolytica* CAB was cultivated in shake flasks with YNB, YPD50, and
3 YPA medium to evaluate the effect of medium composition to cell growth and PHB
4 accumulation (Fig.3 B-D). When glucose was employed as the sole carbon source, the use of
5 complex medium led to significantly higher cell growth than that achieved in defined medium.
6 In YPD medium, cell dry weight reached 24.87 g/l, containing 1.50 % PHB; while the use of
7 defined medium resulted in 4.35 g/l cell dry weight with 2.88 % PHB. When acetate was
8 supplemented as the sole carbon source, the engineered strain produced 6.29 g/l cell dry
9 weight with 3.84 % PHB, which was the highest PHB content.

10 As the synthetic precursor, cytosolic acetyl-CoA is proved to be essential for PHB
11 production. In yeast, there are two potential routes for the generation of cytosolic acetyl-CoA:
12 from citrate by ATP-citrate lyase (ACL), and from acetate *via* acetyl-CoA synthetase (ACS).
13 The citrate is exported from the mitochondrion, while acetate can be externally supplied or
14 generated from acetaldehyde. In *S. cerevisiae*, cytosolic acetyl-CoA is mainly generated from
15 the pyruvate-acetaldehyde-acetate pathway. During glucose fermentation, the majority of
16 carbon flux goes to ethanol, resulting in limited acetyl-CoA availability for biosynthetic
17 pathways (Lian et al. 2014). In *Y. lipolytica*, cytosolic acetyl-CoA is generated from citrate
18 when cultivated on glucose. This flux of acetyl-CoA is likely superior in *Y. lipolytica*
19 compared to *S. cerevisiae* resulting in better PHB producing performance. Moreover,
20 compared with the glucose metabolic pathway, acetate was assimilated and directly converted
21 to acetyl-CoA in cytosol. Thus, the intracellular acetyl-CoA availability would be higher than
22 that obtained from glucose metabolism, leading to the highest PHB content in the three

1 cultivation conditions tested.

2 *3.4 PHB production from acetate in bioreactors*

3 Fermentation was carried out under aerobic conditions using *Y. lipolytica* CAB strain.
4 Acetate was used as sole carbon source and fed by pH control. With defined medium, cell dry
5 weight reached 26.7 g/l with 2.85 g/l PHB accumulation (Fig. 4A). In contrast, complex
6 medium yielded much higher cell growth and PHB accumulation: cell dry weight and PHB
7 titer reached 72.01 g/l and 7.35 g/l, respectively (Fig. 4B). The polymer content was almost
8 the same in both defined medium and complex medium, which suggests that PHB
9 accumulation capacity of engineered *Y. lipolytica* might be restricted by the expression level
10 of heterologous PHB synthetic genes. In previous studies, *E. coli* strain harboring single-copy
11 *phaCAB* operon was unable to accumulate detectable amounts of polymer, and increasing
12 genomic operon copies to 11 led to 5.2 % of cell dry weight PHB production (Yin et al. 2015).
13 In this study, PHB reached approximate by 10 % of cell dry weight in engineered *Y. lipolytica*
14 CAB harboring a single-copy of *phaCAB*. Therefore, in comparison to *E. coli* harboring
15 single-copy *phaCAB* operon, the *Y. lipolytica* CAB constructed here shows superior PHB
16 producing ability.

17 However, the PHB content of engineered *Y. lipolytica* was much lower than that of
18 recombinant *E. coli* harboring high-copy *phaCAB* expression plasmids (Wu et al. 2016a; Wu
19 et al. 2016b). Generally, the cells possess a variety of regulatory mechanisms to maintain
20 intracellular homeostasis. Yeasts are eukaryotic microorganisms and may have much more
21 complicated regulatory mechanisms than that of prokaryotes such as *E. coli*. The flux of
22 carbon toward PHB is probably restricted by the intracellular availability of acetyl-CoA and

1 cofactor pool in the oleaginous yeasts *Y. lipolytica*. Strategies for further increasing PHB
2 production include examining how carbon flux towards the PHB production can be improved
3 by reinforcing the expression level of PHB biosynthetic enzymes, for instance, integrating
4 more copies of *phaCAB* into the genome or optimizing the codon usage. In addition, the
5 native lipids accumulation pathway can be weakened to alleviate the competition of
6 acetyl-CoA precursors and reducing equivalent NADPH.

7 The molecular weight of the PHB produced by *Y. lipolytica* CAB cultivated with acetate
8 in bioreactors was determined by GPC. The weight-average molecular weight ($M_w$) and
9 number-average molecular weight ($M_n$) were $2.0\times10^5$ g/mol and $1.3\times10^5$ g /mol, respectively,
10 which is lower than that of PHB produced by the natural producer *R. eutropha*. The
11 polydispersity index ($M_w/M_n$) was 1.5, indicating narrow molecular weight distributions. It
12 has been proposed that polyhydroxyalkanoate synthase activity and host microorganisms are
13 major determinants controlling the polymer molecular weight and polydispersity (Sim et al.
14 1997). Improving PhaC expression level in *Y. lipolytica* would probably help to increase the
15 molecular weight of the produced PHB.

16 Although acetate is currently produced by fossil-derived methanol carbonylation, it is
17 very abundant in wastewater as a primary component of volatile fatty acids (Chu et al. 1996).
18 Besides, recent studies have demonstrated that acetate can be obtained from waste substrates
19 *via* biochemical processes, for example, anaerobic digestion of food waste (Jiang et al. 2013)
20 or syngas fermentation by acetogenic bacteria *Moorella thermoacetica* from carbon dioxide
21 (Hu et al. 2013). In this regard, compared with glucose, acetate would be a promising cost
22 effective bio-renewable carbon source for microbial fermentation. Our results showed that
23 engineered *Y. lipolytica* can accumulate 7.35 g/l PHB from acetate in 3-l bioreactor

fermentation without process optimization. Further strain engineering and process optimization should be able to develop a low-cost PHB production route.

## 4. Conclusion

In this study, we constructed recombinant *Y. lipolytica* harboring the PHB biosynthetic pathway on its genome. The engineered strain *Y. lipolytica* CAB accumulated 1.50 % and 3.84 % PHB of cell dry weight in shake flask cultures when cultivated with glucose and acetate, respectively. In pH controlled acetate fed-batch fermentation, cell dry weight reached 72.01 g/l with 10.2 % PHB accumulation. This is the first study reporting PHB production from acetate in *Y. lipolytica* and achieved the best performance in terms of PHB production titer in yeast.

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10

1 **Tables**

2 Table 1 Strains and plasmids used in this study

| Name | Description | Reference |
|---|---|---|
| Strains | | |
| *E. coli* NEB-5α | *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | New England Biolabs |
| *Y. lipolytica* Po1g | MATa, leu2-270, ura3-302::URA3, xpr2-3 | Yeastern |
| *Y. lipolytica* CAB | MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2, pTEF-*phaC*, pGPD-*phaA*, pEXP1-*phaB* | This study |
| *Y. lipolytica* C1AB | MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2, pTEF-*phaC1*, pGPD-*phaA*, pEXP1-*phaB* | This study |
| Plasmids | | |
| pQK1 | pGPD, tPOX1, pUC19 backbone | This study |
| pQK3 | pEXP1, tPOX1, pUC19 | This study |
| pMT15 | YLEX php4d::TEFin | (Qiao et al, 2015) |
| pQK1-phaA | *phaA* inserted into pQK1 | This study |
| pQK3-phaB | *phaB* inserted into pQK3 | This study |
| pMT15-phaC | *phaC* inserted into pMT15 | This study |
| pMT15-phaC1 | *phaC1* inserted into pMT15 | This study |
| pMT15-phaCAB | pTEF-*phaC*, pGPD-*phaA*, pEXP1-*phaB* | This study |
| pMT15-phaC1AB | pTEF-*phaC1*, pGPD-*phaA*, pEXP1-*phaB* | This study |

1 Table 2 Oligonucleotides used in this study

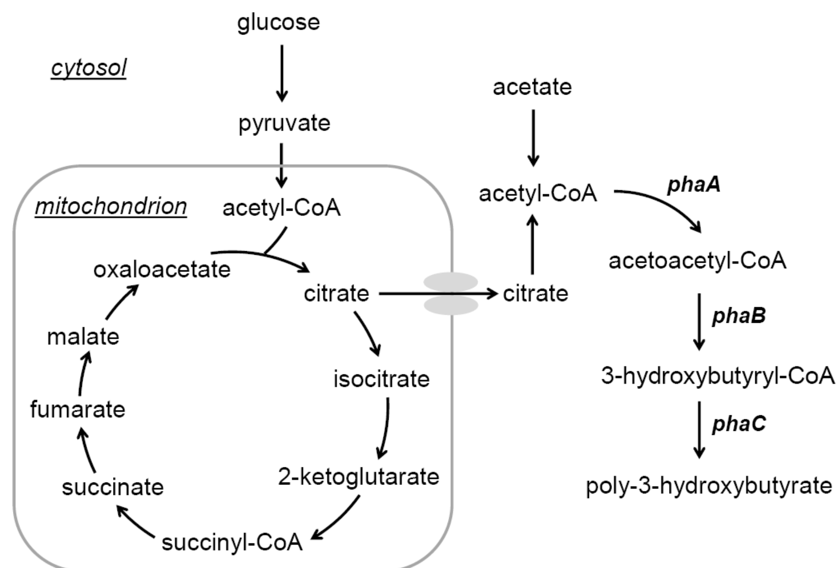
| Primers | Sequence (5’-3’) |
| --- | --- |
| pMT15-phaC-f | CGACCAGCACTTTTTGCAGTACTAACCGCAGGCGACCGGCAAAGGCGCG |
| pMT15-phaC-r | CAAGACCGGCAACGTGGGGTCATGCCTTGGCTTTGACGTAT |
| pMT15-phaC2-f | CGACCAGCACTTTTTGCAGTACTAACCGCAGAGCAACAAAAACAGCGACGACCT |
| pMT15-phaC2-r | CAAGACCGGCAACGTGGGGTTAGCGTTCATGAACATAGGTGCC |
| pQK1-phaA-f | TCTTGAATTAAACACACATCAACAATGACTGACGTTGTCATCGTAT |
| pQK1-phaA-r | TGCATAGCACGCGTGTAGATACTTATTTGCGCTCGACTGCCA |
| pQK3-phaB-f | CACAAGACATATCTACAGCAATGACTCAGCGCATTGCGTA |
| pQK3-phaB-r | CATAGCACGCGTGTAGATACTCAGCCCATATGCAGGCCGC |
| phaCv-phaA-f | AGATGCCCGTGTCCGAATTCCGCAGTAGGATGTCCTGCAC |
| phaA-phaCv-r | GTGCAGGACATCCTACTGCGGAATTCGGACACGGGCATCT |
| phaA-phaB-f | ACAATAAGTCATTCGAGCAAGGTAGGGAGTTTGGCGCCCGTTTTT |
| phaB-phaA-r | AAAAACGGGCGCCAAACTCCCTACCTTGCTCGAATGACTTATTGT |
| phaB-phaCv-f | CAAAGAATGTATCTCATAATTACTAACATTGACAGCTTATCATCGATGATAAGC |
| phaCv-phaB-r | GCTTATCATCGATGATAAGCTGTCAATGTTAGTAATTATGAGATACATTCTTTG |

1 **Figure Legends:**

2 Fig. 1 Poly-3-hydroxybutyrate producing pathway from glucose or acetate in engineered *Y.*

3 *lipolytica*.

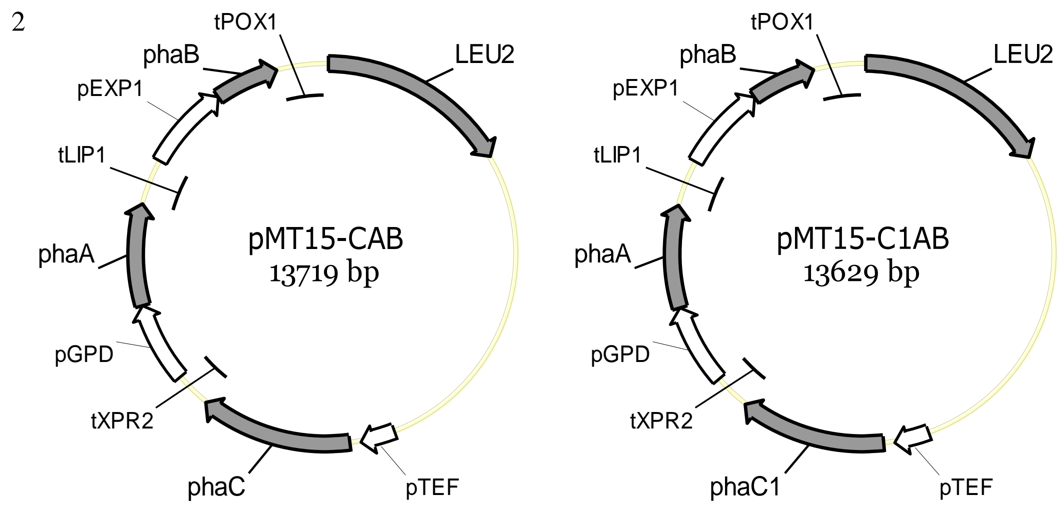
4 Genes: *phaA*, β-ketothiolase; *phaB*, acetoacetyl-CoA reductase; *phaC*, PHA synthase.




5

1 Fig. 2 Schematic maps of plasmids pMT15-CAB and pMT15-C1AB.

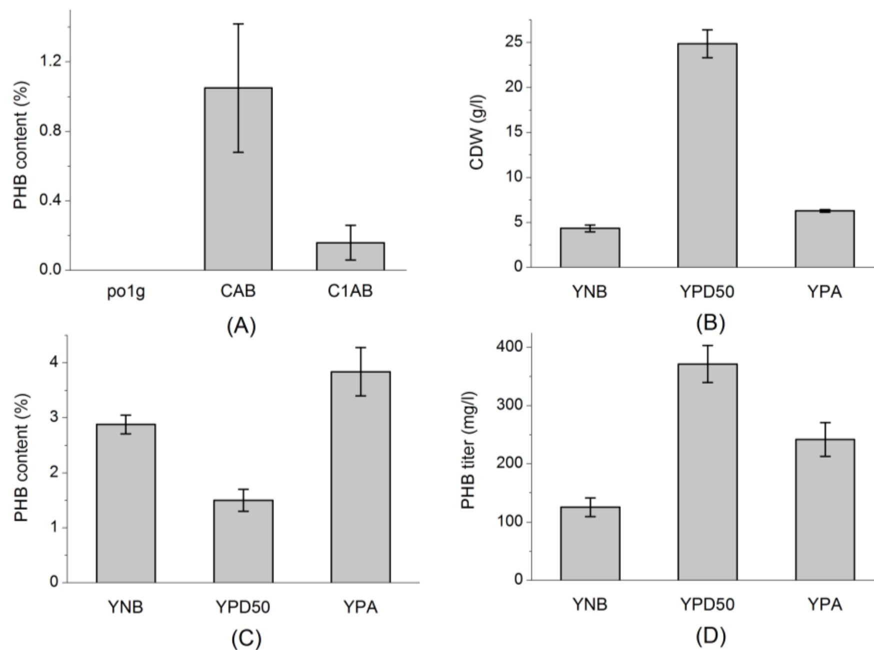
2



1 Fig. 3 PHB production by recombinant *Y. lipolytica* cultivated with different media.

2 (A) Screening of *Y. lipolytica* strains for PHB accumulation in YPD medium.

3 (B)-(D) CDW, PHB content and PHB titer profiles of *Y. lipolytica* CAB grown in shake flasks.

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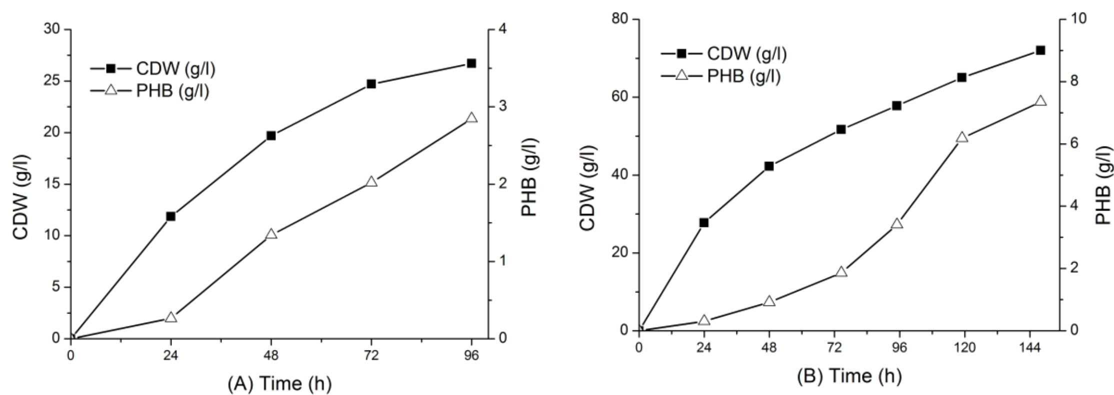
1 Fig. 4 PHB production from acetate by *Y. lipolytica* CAB grown in 3-l bioreactors with

2 defined medium (A) and complex medium (B).




3

4 **Supplementary materials**

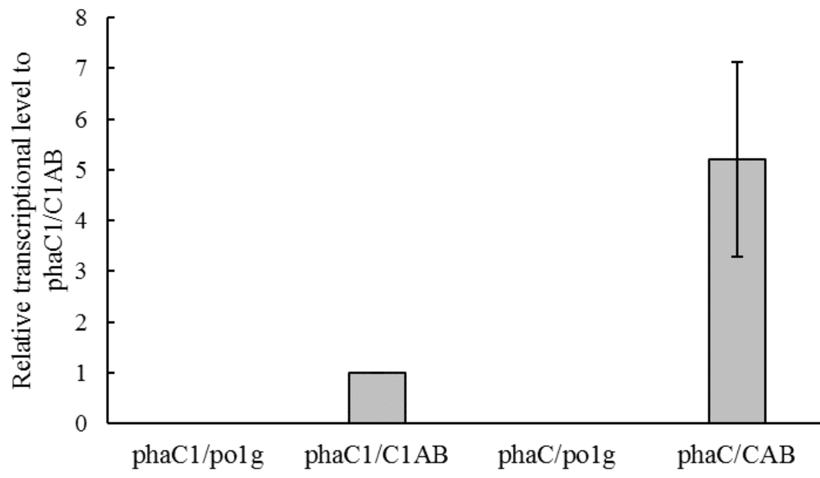
5 Table S1 PCR primers used for transcriptional analysis of *phaC1* and *phaC*.

| Primers | Sequence (5'-) |
|---|---|
| phaC1-RT-F | ATTGATCTGAAACAGGTTA |
| phaC1-RT-R | ATATCGGTAGAGGTCATAT |
| phaC-RT-F | AAGTCCCAACCATTCAAG |
| phaC-RT-R | AGAAGTCCTTCATGTAGC |

6

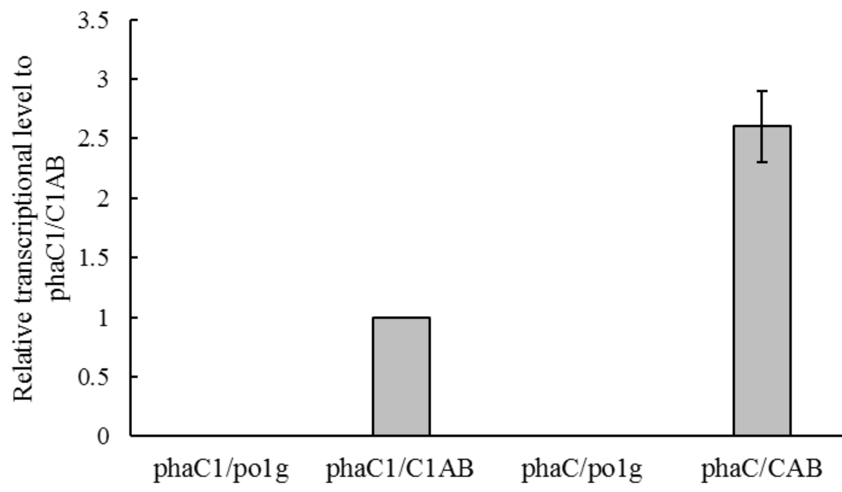
7 Fig. 1 Transcriptional analysis of *phaC1* and *phaC*. *Y. lipolytica* strains were cultivated in

8 YPD medium (A) and YPA medium (B) for 36 h.



(A)

1



(B)

2

3