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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Exploring medium-chain-length polyhydroxyalkanoates production in the engineered yeast *Yarrowia lipolytica*

Cuijuan Gao[1,2,3] · Qingsheng Qi[2] · Catherine Madzak[4,5] · Carol Sze Ki Lin[3]

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Abstract Medium-chain-length polyhydroxyalkanoates (mcl-PHAs) are a large class of biopolymers that have attracted extensive attention as renewable and biodegradable bio-plastics. They are naturally synthesized via fatty acid de novo biosynthesis pathway or β-oxidation pathway from *Pseudomonads*. The unconventional yeast *Yarrowia lipolytica* has excellent lipid/fatty acid catabolism and anabolism capacity depending of the mode of culture. Nevertheless, it cannot naturally synthesize PHA, as it does not express an intrinsic PHA synthase. Here, we constructed a genetically modified strain of *Y. lipolytica* by heterologously expressing *PhaC1* gene from *P. aeruginosa* PAO1 with a PTS1 peroxisomal signal. When in single copy, the codon optimized *PhaC1* allowed the synthesis of 0.205 % DCW of PHA after 72 h cultivation in YNBD medium containing 0.1 % oleic acid. By using a multi-copy integration strategy, PHA content increased to 2.84 % DCW when the concentration of oleic acid in YNBD was 1.0 %. Furthermore, when the recombinant yeast was grown in the medium containing triolein, PHA accumulated up to 5.0 % DCW with as high as 21.9 g/L DCW, which represented 1.11 g/L in the culture. Our results demonstrated the potential use of *Y. lipolytica* as a promising microbial cell factory for PHA production using food waste, which contains lipids and other essential nutrients.

Keywords Medium-chain-length polyhydroxyalkanoates · *Yarrowia lipolytica* · PHA synthase · Strain engineering · Triolein

Introduction

Polyhydroxyalkanoates (PHAs) are a large class of natural biopolymers that is accumulated by microorganisms as a kind of carbon and energy reserves in the presence of an excess carbon source [1, 18]. These polymers have attracted extensive attention because of their thermoplastic and elastomeric properties, making them good candidates for environmentally friendly and biodegradable plastics. There are more than 150 different confirmed types of PHA monomer subunits, each containing varied monomers with different chain lengths/structures, resulting in a wide diversity of material properties [12]. They can be divided into three main types: short-chain-length PHAs (scl-PHAs) which contain repeating units of 3–5 carbon atoms, medium-chain-length PHAs (mcl-PHAs) which contain repeating units of 6–14 carbon atoms, and scl-*co*-mcl PHAs which consist of both SCL and MCL repeating units of 3–14 carbons [6].

Mcl-PHAs are naturally synthesized via fatty acid de novo biosynthesis pathway or β-oxidation pathway

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✉ Carol Sze Ki Lin
carollin@cityu.edu.hk

1 School of Life Science, Linyi University, Linyi 276005, People's Republic of China

2 State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China

3 School of Energy and Environment, City University of Hong Kong, Kowloon, Hong Kong

4 INRA, UMR1319, Micalis Institute, AgroParisTech Campus, CBAI, 78850 Thiverval-Grignon, France

5 Present Address: INRA, UMR 782 Génie et Microbiologie des Procédés Alimentaires, AgroParisTech Campus, CBAI, 78850 Thiverval-Grignon, France

from *Pseudomonads* [28, 29]. Heterologous expression of the key enzymes for mcl-PHA synthesis in a proper host provides a main solution for mcl-PHA synthesis by changing the flux of intermediates of fatty acid metabolism [11, 13, 14, 26, 28, 35]. Heterologous production of mcl-PHA in recombinant *Escherichia coli* and *Saccharomyces cerevisiae* were studied extensively [17, 25, 26, 32].

In the last decades, the unconventional yeast *Y. lipolytica* (originally classified as *Candida lipolytica*) has attracted much interests, notably due to its excellent lipid/fatty acid catabolism and anabolism capacity depending on the cultivation mode [4, 9]. Therefore, it is an interesting microorganism for lipid synthesis or utilization to produce high-value bioproducts [7]. For example, metabolic engineering of *Y. lipolytica* resulted in a strain that produced omega-3 fatty acids eicosapentaenoic acid (EPA) at 15 % of dry cell weight by introducing several heterologous genes, encoding Δ9-elongase, Δ8-desaturase, Δ5-desaturase and Δ17-desaturase, making a breakthrough to replace an animal-derived product [30]. Lipid metabolism was rerouted in this yeast to develop a cell factory for ricinoleic acid (RA) production, capable of accumulating RA to 43 % of its total lipids and over 60 mg/g of cell dry weight [2]. Another effectual work was the synthesis of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) through heterologously expressing linoleic acid isomerase gene from *Propionibacterium acnes*, which was further enhanced by a modified promoter and co-expression of a Δ12-desaturase gene [33, 34]. Recently, Haddouche et al. examined the feasibility of using *Y. lipolytica* for PHA biosynthesis and investigated the roles of multiple acyl-CoA oxidases in the routing of carbon flow towards β-oxidation [10].

Yarrowia lipolytica possesses 16 paralogs of genes coding for lipases which can catalyze the hydrolysis of the ester bond of tri-, di- and mono-glycerides of long-chain fatty acids into fatty acids and glycerol. It has six genes (*POX1–POX6*) encoding isoenzymes of acyl-CoA oxidases (Aoxs) with different substrate specificities that catalyze the first step and also the limiting step of β-oxidation of fatty acids [5, 10]. In contrast, *S. cerevisiae* has only one Aox-encoding gene. Like in *S. cerevisiae*, the β-oxidation cycle of fatty acid of *Y. lipolytica* also provides different length of (*R*)-specific 3-hydroxy-acyl-CoAs (catalyzed by MFE2 type enzyme), which can be used as the direct precursors for PHA synthesis. Hence, it exhibits excellent potential for PHA production. In this study, Mcl-PHA synthase (encoded by *PhaC1*) of *Pseudomonas aeruginosa* PAO1 was heterologously expressed under the recombinant hp4d promoter [21], with a PTS1 peroxisomal signal (SKL). Mcl-PHA production profiles were evaluated, using fatty acid and triolein as substrates.

Materials and methods

Strains and culture conditions

Yarrowia lipolytica strain Po1h and plasmids pINA1312 and pINA1292 have been described previously [21, 23]. *Escherichia coli* DH5α was used for routine subcloning and plasmid propagation. It was grown in Luria–Bertani broth (LB) containing kanamycin (25 mg/L) for plasmid selection. YPD and YNBD media were used for *Y. lipolytica* cultivation and transformants selection, respectively. YPD contained 1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) dextrose. YNBD contained 0.67 % (w/v) yeast nitrogen base (without amino acids and with ammonium sulphate), 0.2 % casamino acids, and 2 % (w/v) dextrose. YNBD containing different concentration of oleic acid (0.1–1.0 %) was used as fermentation substrate by the constructed parallel strains. YPTo and YNBTo (containing 2 % (w/v) triolein instead of dextrose) were also used in mcl-PHA fermentation. Stock solutions of oleic acid (10 % oleic acid, 0.5 % Tween 80) and triolein (25 % triolein, 1.5 % Tween 80) were subjected to sonication three times for 1 min each on ice and heat sterilized separately.

For mcl-PHA production, yeast cells were cultured on YPD plates at 28 °C overnight as the first pre-cultivation step. Then a single colony was picked using a wire loop and aseptically inoculated into a 300 ml flask containing 50 ml of YPD medium and grown at 28 °C for 16–20 h. The cells were harvested by centrifugation and washed once in water and re-suspended in fermentation media with an initial OD_{600} of 0.5. The cultures were then grown at 28 °C, 200 rpm for 72 h before being harvested for mcl-PHA analysis. All the fermentations were done in duplicate.

Plasmids construction

The native and codon-optimized nucleotide sequences of *PhaC1* were cloned and expressed in *Y. lipolytica*. The native coding sequence (GeneBank AE_004091), flanked with *BamH*I and *Kpn*I sites, without and with Kozak sequence, was amplified by PCR using the primers P1 (CCG*GGATCC*ATGAGTCAGAAGAACAATAAC or CCG*GGATCC*GCCACAATGAGTCAGAAGAACAATAAC) and P2 (CGG*GGTACC*CTACAGCTTGGATCGTTCATGCACGTAGGT) on template genome of *P. aeruginosa* PAO1. Italic nucleotides depict the restriction sites and underlined nucleotides are Kozak element. The codon-optimized DNA sequence flanked with *BamH*I and *Kpn*I, with Kozak sequence, was synthesized (Genscript, Nanjing, China). Standard PCR consisted of 15 s at 95 °C, 30 s at 56 °C, and 90 s at 68 °C for 30 cycles. The three versions of DNA fragments digested with the appropriate restriction enzymes were ligated to the same digested pINA1312 and pINA1292

Table 1 *Y. lipolytica* strains constructed for mcl-PHA production

Recombinant strain	Relevant genotype/property	Cloning vector	Source
Po1h	*MatA*, ura3-302, *xpr2-322*, *axp-2* derived from W29 (ATCC24060)		Madzak et al. [21]
PSNC	Po1h containing native *phaC1* gene from *P. aeruginosa* without Kozak sequence	pINA1312	This study
PSC	Po1h containing native *phaC1* gene from *P. aeruginosa* with Kozak sequence	pINA1312	This study
PSOC	Po1h containing codon-optimized *PhaC1* gene with Kozak sequence	pINA1312	This study
PMOC	Po1h containing codon-optimized *PhaC1* gene with Kozak sequence in multiple copies	pINA1292	This study

to form the mono-copy and multi-copy plasmids, respectively. All newly constructed plasmids were screened by restriction enzyme digestion and PCR, and then confirmed by DNA sequencing.

Strain engineering

The expression cassettes based on newly constructed plasmids were linearized using *Not*I enzyme, and introduced into *Y. lipolytica* Po1h to obtain recombinant yeast strains (Table 1). Transformation was performed by the Lithium Acetate Method [3], and transformants were selected by plating on YNBD plates and screened by diagnostic PCR using yeast genome extracted by TIANamp Yeast DNA Kit (TIANGEN, Beijing, China).

Mcl-PHA analysis

Mcl-PHA biosynthesis was confirmed by gas chromatography-mass spectrometry (GC–MS) analysis as described by Zhuang et al. [35]. The ion source temperature was adjusted to 200 °C. The GC analyses were performed as follows: the oven temperature was initially maintained at 60 °C for 3 min, the temperature was gradually increased at a rate of 10 °C per minute up to 250 °C and then held for 6 min, one μl of sample solution was injected. Methyl esters corresponding to mcl-PHA repeating monomers were determined based on retention time and fragmentation patterns of known standards and resulting mass spectra available compared with GC–MS library database (NIST08s). The MS was operated in scanning mode between 20 and 350 *m/z*.

The content and monomer compositions of intracellular accumulated mcl-PHA were analyzed by gas chromatography (GC). Its content was defined as the percentage ratio of mcl-PHA concentration to dried cell weight (DCW). Liquid culture was harvested by centrifugation, washed twice in water and lyophilized. Lyophilized cells were extracted four or five times with warm methanol (65 °C) to remove lipids, free fatty acids and acyl-CoA, including 3-hydroxyacyl-CoA, while PHA (insoluble in methanol) remained associated with the cells [11]. After centrifugation and removal of the residual methanol, the material (15 mg) was subjected to methanolysis in the presence of 1 ml of chloroform and 1 ml of 3 % (v/v) sulfuric acid in methanol for 1 h at 100 °C. The samples were cooled to room temperature and then 1 ml of distilled water was added in order to extract the cell debris, soluble in the aqueous phase. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min. After layer separation, the organic (chloroform) phase (500 μl) was transferred to another new vial and analyzed using a Shimadzu GC2010 gas chromatograph (Kyoto, Japan) equipped with an AOC-20i auto-injector and a RestekRxi®-5 column. PHA standard samples (Sigma-Aldrich) were also analyzed by GC according to the method above. The temperature program used was as follows: 60 °C held for 3 min, ramped from 60 to 260 °C at 10 °C min^{-1} and then held for 6 min.

Results

Engineering of *Y. lipolytica* for mcl-PHA biosynthesis

PhaC1, encoding a mcl-PHA polymerase from *P. aeruginosa* PAO1, was heterologously expressed with a PTS1 peroxisomal signal to ensure its proper expression in peroxisomes of *Y. lipolytica*. Since the codon usage of native *PhaC1* sequence differs from that preferred by *Y. lipolytica*, the gene was codon-optimized as well. To test the effectiveness of codon optimization in the expression of *PhaC1*, both the native gene (*PhaC1*) and the codon-optimized gene (*oPhaC1*) were inserted into plasmid-borne expression cassettes driven by a strong promoter hp4d. Additionally, the Kozak element was added before the first AUG codon to prevent the leaky scanning of the ribosome and increase the efficiency of translation [8]. Expression cassettes were derived from single-copy vector pINA1312 which contains the non-defective *ura3d1* gene for mono-copy expression [23]. Three expression cassettes were constructed and used to transform *Y. lipolytica* after being linearized by *Not*I, resulting in three parallel mcl-PHA producing strains: PSNC, PSC and PSOC (Table 1). Recombinant yeast strains harboring these variant versions

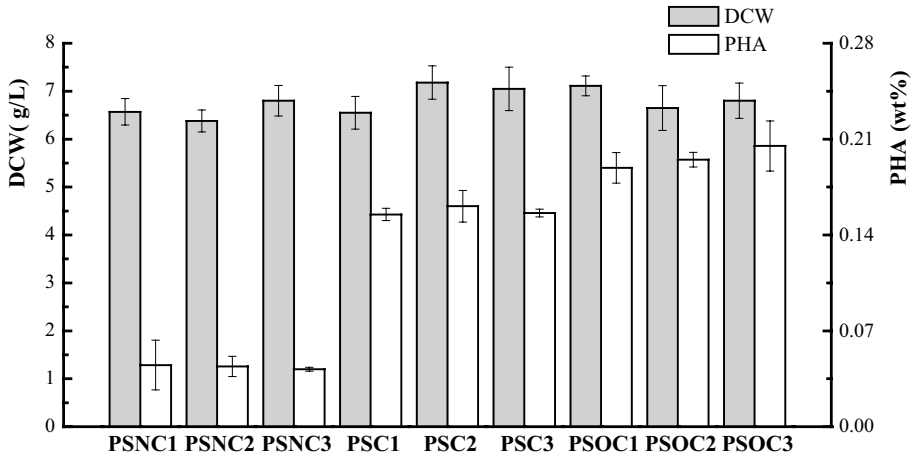

Fig. 1 Biomass and mcl-PHA accumulation in 3 transformants of the strains PSNC, PSC and PSOC. wt%, the percentage of mcl-PHA content in the cell dry weight

of PhaC1 expression cassette were fermented in YNBD media containing 0.1 % oleic acid. Three transformants of each recombinant strain were picked randomly for PHA fermentation. GC–MS results showed that the engineered strain harboring mcl-PHA synthase accumulated mcl-PHA compared with the control strain of Po1h (Fig. S1). PHA yields in the different recombinant *Y. lipolytica* strains are shown in Fig. 1. All engineered strains grew to a similar biomass (approximately 7 g/L DCW) and produced mcl-PHA in yields that were significantly different between the constructs, but similar between the 3 transformants for the same construct. The 3 PSNC clones, without Kozak element before the initial AUG codon of native *phaC1* gene, produced the least amount of PHA (mean yield of 0.044 % of dried cell weight (DCW)). In contrast, the 3 PSC clones, with the Kozak element added upstream of native *phaC1* gene, produced a mean yield of 0.157 % of PHA, which is 3.6-fold that produced by PSNC. Furthermore, the combined effect of codon-optimization and addition of Kozak sequence in the 3 PSOC clones resulted in an average PHA yield of 0.205 %, 4.7-fold more than that of PSNC.

Integration of *PhaC1* in multiple copies

As shown in Figs. 1 and 2, exogenous PhaC introduced into peroxisomes of *Y. lipolytica* polymerized 3-hydroacyl-CoA, which is an intermediate of MFE2 of β-oxidation, to form mcl-PHA. Using codon-optimized gene (*oPhaC1*) and Kozak sequence, a relatively high PHA yield was obtained. Accordingly, enhanced activity of PhaC can seize more intermediates from β-oxidation and yield higher PHA accumulation. This allows further strengthening of the *PhaC1* expression using a multiple integration strategy. The multi-copy vector pINA1292, which contains the defective *ura3d4* gene and is required in multiple copies to alleviate the uracil auxotrophy of the host, was adopted to develop a more efficient PHA-producing strain [23]. Three individual transformants were picked randomly to test their abilities for mcl-PHA production, using PSOC as a control strain. As shown in Fig. 3, both DCW and PHA yields were improved. Accordingly, for the multi-copy strain, PHA yields appeared to be slightly but significantly different between PMOC1 and PMOC3 transformants (Fig. 3). PMOC1 strain was the best PHA producer, with a yield of 1.46 %, sevenfold more than that of PSOC strain. The average mcl-PHA contents and DCW of PMOCs reached 1.28 % and 8.3 g/L respectively after 72 h cultivation in a shake flask with YNBD medium containing 0.1 % oleic acid.

Optimizing cultivation for mcl-PHA production

Increasing mcl-PHA content by raising the concentration of oleic acid

In order to explore whether the ability of PHA biosynthesis can be further enhanced, fermentation using YNBD medium contained various concentrations of oleic acid from 0.1 to 1.0 % were carried out using the best-performing strain of PMOC. The biomass and mcl-PHA accumulation are presented in Table 2. As we expected, more oleic acid in the culture can lead to higher mcl-PHA accumulation, although its increase is not completely linear, especially when the levels of oleic acid increased from 0.5 to 1.0 %. The highest mcl-PHA was 0.43 g/L in YNBD with 1 % oleic acid. The composition of mcl-PHA monomers is listed in Table 2. In all conditions, (*R*)-3-hydroxyoctanoate (3HO) was the most abundant monomer in the final polyester, accounting for 41-48.4 mol% of the monomer composition. It indicates a specificity of the *P. aeruginosa* PHA synthase for (*R*)-3-hydroxy-octanoyl-CoA.

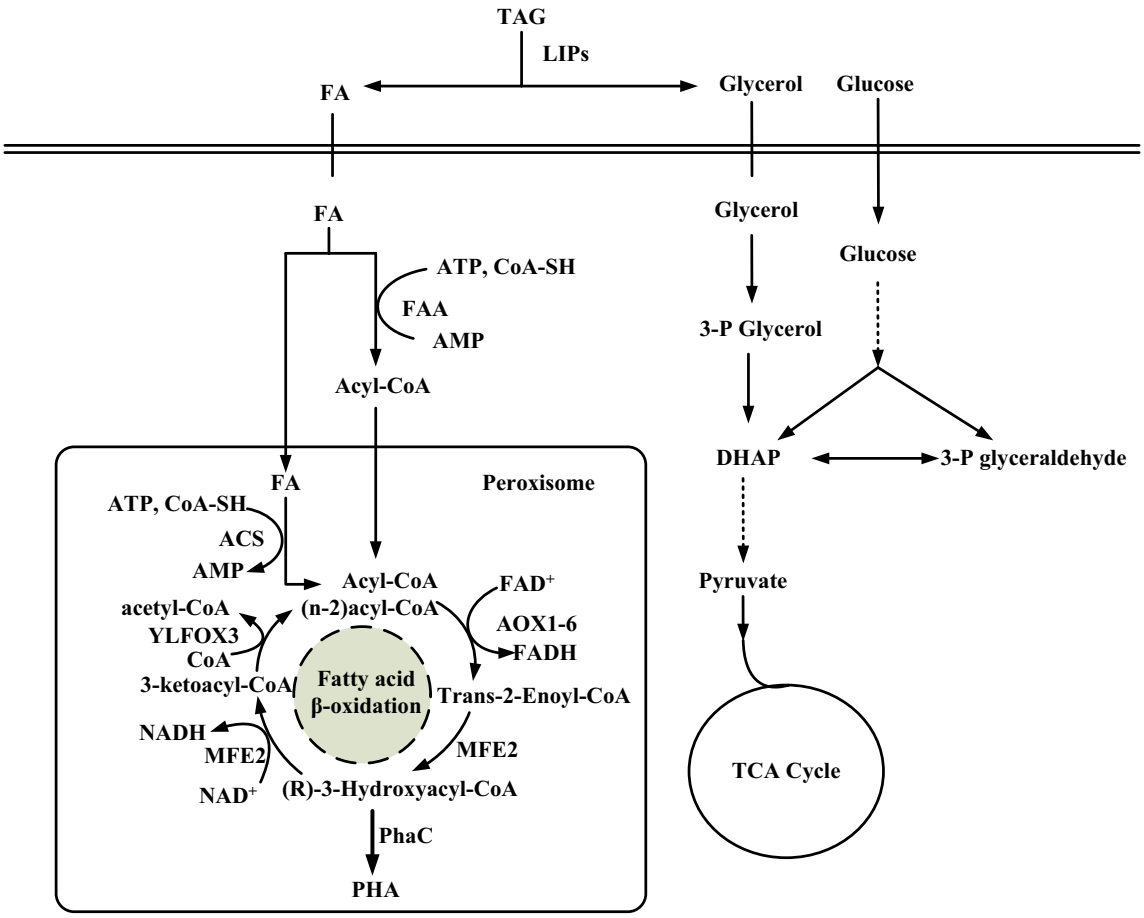

Fig. 2 Schematic pathway involved in the key routes of modified catabolism of lipids, fatty acid, glycerol and glucose. LIPs, lipases; FAA, acyl-CoA synthetase; POXAOX, acyl-CoA oxidase; MFE2, a multifunctional enzyme with one 2-enoyl-CoA hydratase domain and two *R*-3-hydroxyacylCoA dehydrogenase domains; YLFOX3, 3-ketoacyl CoA thiolase

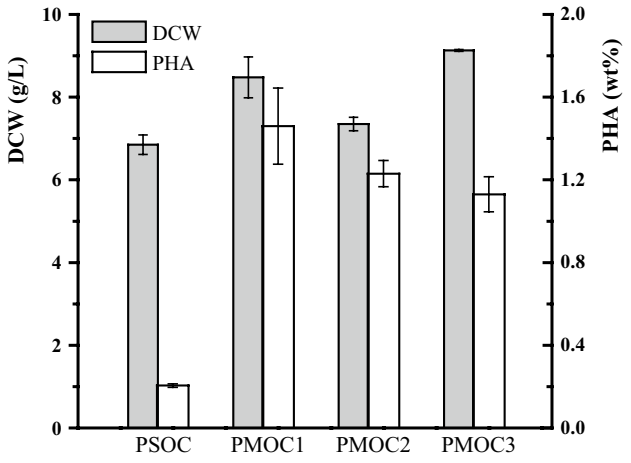

Fig. 3 Biomass and mcl-PHA accumulation in the strain PSOC (mean values for the 3 transformants) and in 3 transformants of the strain PMOC. wt%, the percentage of mcl-PHA content in the cell dry weight

Increased mcl-PHA content by utilizing triolein as substrate

Since fatty acids are usually expensive, toxic to cells and insoluble (poorly miscible) in aqueous solution, glucose needs to be added as a supplementary carbon source to ensure better growth of yeast cells. In this study, triolein, as a representative triacylglycerol, was employed as single substrate for mcl-PHA synthesis in YPTo and YNBTo medium. As shown in Table 3, the PHA composition using triolein as substrate is consistent with that in YNBD medium containing oleic acid, while the PHA content increased to over 5.0 % DCW. Moreover, rich medium (YPTo) can yield higher biomass (21.9 g/L), which resulted in an accumulation of 1.11 g/L mcl-PHA. Thus, triolein constitutes an alternative and cheaper carbon source for PHA production.

Table 2 Content and composition of mcl-PHAs synthesized by recombinant *Y. lipolytica* PMOC in YNBD with different concentrations of oleic acid

Oleic acid contents (%)	DCW (g/L)	PHA (% DCW)	PHA (g/L)	3HHx (mol%)	3HO (mol%)	3HD (mol%)	3HDD (mol%)	3HTD (mol%)
0.1	9.13	1.13 ± 0.051	0.10	6.3	42.9	27.9	18.5	4.5
0.2	9.10	1.85 ± 0.036	0.17	5.9	41.0	24.8	16.0	12.1
0.5	12.00	2.58 ± 0.126	0.31	6.4	44.8	26.1	16.9	5.8
1.0	14.95	2.84 ± 0.142	0.43	7.3	48.4	25.0	15.6	3.6

DCW dried cell weight, *3HHx* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *3HD* 3-hydroxydecanoate, *3HDD* 3-hydroxydodecanoate, *3HTD* 3-hydroxytetradecanoate

Table 3 Content and composition of mcl-PHAs synthesized by recombinant *Y. lipolytica* PMOC with 2 % triolein as substrate in YNBTo and YPTo

Medium	DCW (g/L)	PHA (% DCW)	PHA (g/L)	3HHx (mol%)	3HO (mol%)	3HD (mol%)	3HDD (mol%)	3HTD (mol%)
YNBTo	8.72	5.13 ± 0.30	0.45	7.0	48.4	26.6	16.2	1.8
YPTo	21.9	5.05 ± 0.54	1.11	7.8	45.9	26.4	17.7	2.3

Discussion

Mcl-PHAs are a large class of biopolymers that are attractive for a wide range of potential applications. The aim of this study was to apply metabolic engineering to the oleaginous yeast *Y. lipolytica* for the production of mcl-PHA. Yeast does not naturally synthesize PHAs, as it does not express an intrinsic PHA synthase. However, it can provide the direct precursors, different length of (*R*)-specific 3-hydroxy-acyl-CoAs, by β-oxidation of fatty acids that can be used as substrates for mcl-PHA biosynthesis. Mcl-PHA was previously synthesized in *S. cerevisiae* transformed with heterologous PHA synthase targeted to peroxisomes, using even-chain length and odd-chain length fatty acids [25, 32]. The maximum amount of PHA accumulated was 0.45 % DCW [25]. Thereafter, mcl-PHA was similarly synthesized in peroxisomes of *Pichia pastoris*, and reached 1 % DCW using oleic acid as substrate [24]. Recently, *Y. lipolytica* was engineered for synthesis of mcl-PHA, which was further improved by inactivating the R-3-hydroxyacyl-CoA dehydrogenase domain of MFE2 and blocking the neutral lipid synthesis pathway using 0.2 % tridecanoic acid in YNB minimal medium [10, 11]. These studies indicated that *Y. lipolytica*, which exhibits efficient lipid/fatty acid catabolism and anabolism capacities, owns a tremendous potential for mcl-PHA synthesis in comparison with other yeasts.

In this study, a series of recombinant *Y. lipolytica* strains was constructed for mcl-PHA production. PHA compositions remained consistent in the different strains constructed and with different carbon sources (Tables 2, 3). In all conditions, (*R*)-3-hydroxyoctanoate (3HO) was the most abundant monomer in the final polyester, accounting for 41–48.4 mol% of the monomer composition. This reflects the specificity of the *P. aeruginosa* PHA synthase for R-3-hydroxy-octanoyl-CoA, as proposed previously [22]. PHA content of PSC is 3.6-fold that of PSNC (Fig. 1), indicating that the inclusion of Kozak element increased gene expression in this study. Strain PSOC with codon optimized *PhaC1* gene further increased mcl-PHA accumulation. With multiple integration of codon-optimized *PhaC1*, PMOC output was of 5.0 % and 1.11 g/L of PHA in the culture using triolein as the sole substrate in rich medium (YPTo), which ensured an excellent cell growth and a high resultant cell dry weight (22.0 g/L DCW).

To synthesize chemicals heterologously through strategies of metabolic engineering tends to encounter the issue of the competition between product titers and yields. To this end, improved PHA production is usually accompanied by a decrease in cell growth. In the studies of PHA synthesis, fatty acids were commonly used as substrates. However, fatty acids are insoluble and toxic to cells in aqueous solution, which led to poor cell growth. A solution for that is adding other carbon sources, such as glucose or glycerol, as co-substrates to ensure proper cell growth and biomass production. Therefore, a more balanced genetic method (evolutionary approach, for example) should be taken further to improve the ability of engineered strains. Otherwise, strategies like culture optimization using different sources of feedstocks can be applied. Herein, triolein turned out to be an excellent carbon source for mcl-PHA production, which yielded both high product concentration and biomass. Besides, *Y. lipolytica* used in our study was not modified for its lipid and fatty acid metabolism at all. Future works, including deletion

of the neutral lipid synthesis pathway and overexpression of AOXs charging rate-limited step of β-oxidation, which might further improve the yield of mcl-PHA, are currently planned for investigation in our laboratory.

Sustainable valorization of renewable feedstock can produce value-added chemicals and biopolymers via microbial bioconversion [15, 19]. As described in our recent study, food waste is a serious global issue [27]. It contains various nutrients such as sugars, free amino nitrogen (FAN) and lipids which can be released after hydrolysis and separation. Food waste-based biorefinery, as a novel concept, received a significant attention in recent years [16, 20]. In comparison with other yeast, *Y. lipolytica* has excellent potential for utilising hydrophobic substrates efficiently. Our recent studies successfully demonstrated the use of glucose and FAN as major nutrients from food waste for microbial production of high value-added products such as succinate [27, 31]. Our future studies will target the full exploration of lipid fraction from food waste to produce PHA using engineered *Y. lipolytica*, as this study demonstrated the novel use of triolein for PHA synthesis.

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