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Control of D-lactic acid content in P(LA-3HB) copolymer in the yeast Saccharomyces cerevisiae using a synthetic gene expression system

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ABSTRACT

The fully bio-based polyhydroxyalkanoate (PHA) polymers provide interesting alternatives for petrochemical derived plastic materials. The mechanical properties of some PHAs, including the common poly(3-hydroxybutyrate) (PHB), are limited, but tunable by addition of other monomers into the polymer chain. In this study, we present a precise synthetic biology method to adjust lactate monomer fraction of a polymer by controlling the monomer formation in vivo at gene expression level, independent of cultivation conditions. We used the modified doxycycline-based Tet-On approach to adjust the expression of the stereospecific D-lactate dehydrogenase gene (ldhA) from Leuconostoc mesenteroides to control D-lactic acid formation in yeast Saccharomyces cerevisiae. The synthetic Tet-On transcription factor with a VP16 activation domain was continuously expressed and its binding to a synthetic promoter with eight transcription factor specific binding sites upstream of the ldhA gene was controlled with the doxycycline concentration in the media. The increase in doxycycline concentration correlated positively with ldhA expression, D-lactic acid production, poly(D-lactic acid) (PDLA) accumulation in vivo, and D-lactic acid content in the poly(D-lactate-co-3-hydroxybutyrate) P(LA-3HB) copolymer. We demonstrated that the D-lactic acid content of the P(LA-3HB) copolymer can be adjusted linearly from 6 mol% to 93 mol% in vivo in S. cerevisiae. These results highlight the power of controlling gene expression and monomer formation in the tuning of the polymer composition. In addition, we obtained 5.6% PDLA and 19% P(LA-3HB) of the cell dry weight (CDW), which are over two- and five-fold higher accumulation levels, respectively, than reported in the previous studies with yeast. We also compared two engineered PHA synthases and discovered that in S. cerevisiae the PHA synthase PhaC1437ps619 produced P(LA-3HB) copolymers with lower D-lactic acid content, but with higher molecular weight, in comparison to the PHA synthase PhaC1Pre.

1. Introduction

The global awareness of plastic waste management issues has increased the demand for novel environmentally friendly materials. Native and engineered microorganisms are able to produce biobased, biodegradable, and thermoformable poly(hydroxyalkanoate) (PHA) polymers, including poly(3-hydroxybutyrate) (PHB) (Peoples and Sinskey, 1989a; 1989b) and many different short- and medium chain length PHAs (Choi et al., 2020). In addition, protein engineering of few PHA synthases (Jung et al., 2010; Taguchi et al., 2008; Yang et al., 2010) and propionyl-CoA transferases (Prabhu et al., 2012; Taguchi et al., 2008; Yang et al., 2010) has enabled production of poly(D-lactic acid) (PDLA) in vivo.

The mechanical properties of some homopolymeric PHAs are rather limited, but tunable by incorporation of other monomers into the polymer chain. For example, the presence of only 15 mol% of D-lactic acid in the poly(D-lactate-co-3-hydroxybutyrate) copolymer (P(LA-3HB)) increases the polymer flexibility over 8-fold and decreases the melting temperature by 10 °C (Yamada et al., 2011), in comparison to PHB. The lower melting temperature facilitates material processing below the thermal decomposition temperature. The increased flexibility and lower melting temperatures are not limited to P(LA-3HB) copolymer, but similar phenomena are observed also with PHA copolymers containing 3-hydroxypropionate and 4-hydroxybutyrate monomers (Doi et al., 1990; Li et al., 2010; Meng et al., 2012). In fact, a recent PHA modelling study, focusing on predicting the glass transition temperature (Tg) of different PHA copolymers, reports that the relative amount of two different monomers is the second most important parameter defining the Tg, after the choice of the monomer (Jiang et al., 2020).

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These findings emphasise the importance of controlling the monomer ratios in development of the new PHA copolymers.

Production of the P(LA-3HB) copolymer has been studied earlier in vitro and in vivo in two bacterial strains, Escherichia coli and Corynebacterium glutamicum, as reviewed in (Choi et al., 2020), and most recently in vivo by us in the yeast Saccharomyces cerevisiae (Ylinen et al., 2021). In previous studies polymers with D-lactic acid contents from 3 to 30 mol% (Kadoya et al., 2015), 20–50 mol% (Yang et al., 2010), 8–73 mol% (Nduko et al., 2014), and from 55 to 86 mol% (Jung et al., 2010), were obtained by controlling physiological parameters such as aeration and monomer feeding, and by expressing different PHA synthases. In the yeasts S. cerevisiae and Yarrowia lipolytica physiological parameters, substrate feeding, and changes in β-oxidation were used for controlling monomer content in medium chain length (mcl) and short chain length (scl) PHA copolymers (De Oliveira et al., 2004; Gao et al., 2015; Had douche et al., 2010, 2011; Poirier et al., 2001; Rigouin et al., 2019; Zhang et al., 2006). However, none of the studied methods is readily applicable for ubiquitous control of production of other monomers in vivo. This would be highly important when novel monomers are too expensive, toxic, or unavailable for feeding in sufficient concentrations. We wanted to study tuning of monomer ratios at the level of gene expression to control polymer properties independent of cultivation conditions. So far, this has been studied relatively little and only in bacterial species. Only one study in E. coli exploits the possibility to induce gene expression of 3-hydroxybutyrate-CoA (3HB-CoA) related genes with different arabinose concentrations (Wang et al., 2013). Results from that study are however encouraging with a wide range (10–95 mol%) of 3-hydroxypropionate in PHB backbone. The remaining few bacterial studies rely on building of large strain libraries with different promoters or ribosome binding sites, or on CRISPRi based repression of gene expression, and report only smaller monomer ratios of 0–13 mol% or 0–45 mol% (3-hydroxyvalerate, 4-hydroxybutyrate, or 3-hydroxyhexanoate) in PHB backbone (Arikawa et al., 2016; Lv et al., 2015; Tao et al., 2017; Yu et al., 2020).

The PHA production in yeasts has not been studied as widely as in bacterial strains. However, different yeast species offer interesting options for the PHA production being capable of growing on different inexpensive substrates and tolerating acidic conditions (Rigouin et al., 2019). In addition, yeasts lack endotoxins and pharmaceutic properties. Only one study in the bacterial strain Cupriavidus necator (Leaf et al., 1996). Resulting strain accumulated 0.5% PHB of cell dry weight (CDW). PHB accumulation was later increased in S. cerevisiae to approximately 9% of CDW by introduction of entire PHB pathway from C. necator, including genes for acetyl-CoA acetyltransferase (phaA) and acetoacetyl-CoA reductase (phaB1) (Carlson and Srienc, 2006), and up to 14–16.4% of CDW when phaB1 was replaced with gene encoding NADH dependent acetoacetyl-CoA reductase variant from Allocromatium vinosum and strains were grown on xylose in anaerobic conditions (de Las Heras et al., 2016; Portugal-Nunes et al., 2017). Engineering of PHB and mcl-PHA production in other yeasts has resulted in accumulation of up to 25%, 30%, and 52% PHAs of CDW in yeasts Y. lipolytica, Pichia Pastoris, and Arxula adeninivorans, respectively (Biernacki et al., 2017; Rigouin et al., 2019; Vijayasankaran et al., 2005). In our previous study we engineered the yeast S. cerevisiae for the production of PDLA, PHB, and their copolymer P(LA-3HB) (Ylinen et al., 2021). We obtained D-lactic acid contents of 46–65% in the copolymer P(LA-3HB) by expressing a stereospecific D-lactate dehydrogenase gene (ldhA) from Leuconostoc mesenteroides from a constitutive pTDH3 promoter.

Here we studied the possibility to adjust the P(LA-3HB) monomer composition at the gene expression level by regulating the D-lactic acid production in vivo. The expression of the ldhA gene was adjusted with a doxycycline controlled Tet-On expression system (Kakko et al. in preparation; Belli et al., 1998; Gossen et al., 1995; T. Das et al., 2016), which was modified based on our previously constructed synthetic expression system (SES), where the synthetic transcription factor controls expression of a synthetic promoter consisting of a varying number of binding sites followed by a short core promoter (Rantasalo et al., 2018a). The controlled D-lactic acid production inspired us also to study how the D-lactic acid availability affects the PDLA accumulation in the cells. In addition, two engineered PHA synthases and their different copy numbers were compared for their efficiencies in the D-lactyl-CoA polymerization in S. cerevisiae.

2. Materials and methods

2.1. Strains and plasmids

The studied genes, plasmids, and oligos are listed in Table 1 and the yeast strains in Table 2. The parent strain, haploid S. cerevisiae strain CEN.PK11-9A (H3892), with LEU2 and HIS3 auxotrophies, was kindly provided by Dr. P. Kötter from Institut für Mikrobiologie (J.W. Goethe Universität Frankfurt, Germany). Plasmids were cloned using Gibson Assembly (E2611S, New England BioLabs) and E. coli TOP10 cells. The lithium acetate method (Gietz and Schiestl, 2007) was used for all yeast transformations.

The endogenous D-lactate dehydrogenase gene (LDL1) of the strain CEN.PK11-9A was deleted with simultaneous integration of codon optimized, stereospecific D-lactate dehydrogenase from L. mesenteroides (ldhA) (Baek et al., 2016; Ylinen et al., 2021). Deletion was carried with CRISPR/Cas9 method using two gRNA plasmids B11839 and B11840 presented in Table 1. The ldhA gene was expressed either under a constitutive pTDH3 promoter or a doxycycline controllable Tet-On system (Fig. 1) (Belli et al., 1998; Gossen et al., 1995; T. Das et al., 2016).
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2.2.1. Cell growth study in bioscreen C equipment

The cell growth with doxycycline concentrations of 0, 1, 5, and 10 mg L$^{-1}$ was followed every 10 min in Bioscreen C equipment in multi-well plates at 30 °C using 200 μL total volume of the media and a starting OD$_{600}$ of approximately 0.10–0.11. The starting OD$_{600}$ was measured with VitroSpec 2100 Pro equipment (Amersham Biosciences), but later the growth was followed with the Bioscreen spectrophotometer directly with VitroSpec 2100 Pro equipment (Amersham Biosciences), but later the growth was followed with the Bioscreen spectrophotometer directly (Kakko et al. in preparation) and biological replicates. The outer wells were filled with water to decrease the media evaporation from the sample wells.

2.2.2. Polymer extraction and analysis

The polymers were analyzed as described previously (Ylinen et al., 2021). The cell growth was followed as optical density (OD$_{600}$), or as CDW. When necessary, the OD$_{600}$ values for the strain phaC1Pre_1x were converted to CDW with a linear correlation function (OD$_{600}$ = 3.9822CDW$^{0.9895}$, R$^2$ = 0.98) derived from another experiment where the same strain was grown in similar conditions (Supplemental Fig. S1).

Table 1

The studied genes, plasmids, and oligos.

| Genes | Description | Reference |
|-------|-------------|-----------|
| phaA  | Acetyl-CoA acetyltransferase from Cupriavidus necator, GenBank KP681582 | Sandstrom et al. (2015) |
| phaB1 | Acetoacetyl-CoA reductase from C. necator, GenBank KP681583 | Sandstrom et al. (2015) |
| idhA  | Stereospecific D-lactate dehydrogenase (IdhA) from Leuconostoc mesenteroides | Bæk et al. (2016) |
| pctMe | Propionyl-CoA transferase from Megasphaera elsdenii, European Nucleotide Archive ERZ1065933 | Prabhu et al. (2012) |
| phaC1437ps6-19 | PHA synthase from Pseudomonas sp. MBEL 6–19, with amino acid substitutions E130D, S325T, S477G, and Q481K | Yang et al. (2010) |
| phaC1Pre | PHA synthase from Pseudomonas respiratorius, with amino acid substitutions E130D, S325T, S477G, and Q481K | Yang et al. (2011) |

Table 2

Plasmids

| Name | Description | EasyClone integration locus | Reference |
|------|-------------|-----------------------------|-----------|
| B11841 | pTEF1-pcMe-tCYC | X-3 | This article |
| B11843 | pTDH3-phaC1Pre-tCYC | X-4 | This article |
| B11847 | pTDH3-phaC1Pre-tCYC | XII-5 | This article |
| B11848 | pTDH3-phaC1Pre-tCYC | XI-5 | This article |
| B11844 | pTDH3-phaC1437ps6-19-tCYC | X-4 | This article |
| B11845 | pTDH3-phaC1437ps6-19-tCYC | XII-5 | This article |
| B11846 | pTDH3-phaC1437ps6-19-tCYC | XI-3 | This article |
| B11849 | pTDH3-phaC1437ps6-19-tCYC | X-2 | This article |
| B11850 | pTDH3-phaC1437ps6-19-tCYC | XII-4 | This article |
| B11851 | pTDH3-phaC1437ps6-19-tCYC | XI-5 | This article |
| B11852 | pTDH3-phaC1437ps6-19-tCYC | X-2 | This article |
| B11853 | pTDH3-phaC1437ps6-19-tCYC | XII-4 | This article |
| B11854 | pTDH3-phaC1437ps6-19-tCYC | XI-5 | This article |
| B11855 | pTEF1-phaC1Pre-tCYC | XI-1 | This article |
| B9091 | pTDH3-phaC1Pre-tCYC | 60 bp flank to phaC1Pre_1x | (Kakko et al. in preparation) |

Oligos

| Name | Sequence | Reference |
|------|----------|-----------|
| UBGC1qPCR_F (1189) | ACTTTCCCCTCGTCTGATATCCA | Rantasalo et al. (2018b) |
| UBGC1qPCR_R (1190) | TAATTGATCCTGTCGTGGCT | Rantasalo et al. (2018b) |
| lhdA1lqPCR_F | ATGCCATAAGTCGTCGTGACCC | This article |
| lhdA1lqPCR_R | AAATCTACTTCCACCTCAGTC | This article |
| lhdA2lqPCR_F | AGAGTGGCGAATAAACATCT | This article |
| lhdA2lqPCR_R | GCCGTCTATATCCCATGATTACC | This article |

containing Tet-On synthetic transcription factor (sTF) described by Kakko et al. (in preparation), eight binding sites for the sTFs (Rantasalo et al., 2018a), and an ENO1 core promoter (ENO1cp) (Rantasalo et al., 2018a). The ENO1cp is a minimal sequence required for initiation of the core promoter (ENO1cp) and is integrated into the yeast strains using CRISPR/Cas9 technology and EasyClone gRNA vectors (Jespøl-Fabre et al., 2016) for the selection.

2.2. Culture conditions and analytical methods

E. coli was grown in Luria–Bertani medium containing either ampicillin (100 μg/mL) or kanamycin (50 μg/mL). The yeast strains were grown in 50 mL of synthetic complete media supplemented with 20 g L$^{-1}$ glucose and 0–10 mg L$^{-1}$ doxycycline in three replicates for 24–72 h at 30 °C with 220 rpm shaking. The cell growth, pH, and metabolite production were analyzed daily as described previously (Ylinen et al., 2021). The cell growth was followed as optical density (OD$_{600}$), or as CDW. When necessary, the OD$_{600}$ values for the strain phaC1Pre_1x were converted to CDW with a linear correlation function (OD$_{600}$ = 3.9822CDW$^{0.9895}$, R$^2$ = 0.98) derived from another experiment where the same strain was grown in similar conditions (Supplemental Fig. S1).

2.2.1. Cell growth study in bioscreen C equipment

The cell growth with doxycycline concentrations of 0, 1, 5, and 10 mg L$^{-1}$ was followed every 10 min in Bioscreen C equipment in multi-well plates at 30 °C using 200 μL total volume of the media and a starting OD$_{600}$ of approximately 0.10–0.11. The starting OD$_{600}$ was measured with VitroSpec 2100 Pro equipment (Amersham Biosciences), but later the growth was followed with the Bioscreen spectrophotometer directly from the multiwell plates. The samples were analyzed in three technical and biological replicates. The outer wells were filled with water to decrease the media evaporation from the sample wells.

2.2.2. Polymer extraction and analysis

The polymers were analyzed as described previously (Ylinen et al., 2021). The cells were washed with distilled water three times and lyophilized over night. The quantity and the composition of the accumulated polymers were analyzed directly from the lyophilized cells with gas chromatography–mass spectrometry (GC-MS). The polymers were extracted from the lyophilized cells by boiling the cells in 95 °C chloroform for 3 h followed by filtration through 0.45 μm PTFE filters and two non-solvent precipitation washing steps. The phospholipids were removed with methanol and the neutral lipids with diethyl ether. The molecular weights of the extracted polymers were analyzed with a
chloroform based size exclusion chromatography (SEC).

2.2.3. Transcriptional analysis

The transcription analysis was carried as described previously (Rantasalo et al., 2018a). Two oligo pairs (Table 1) were used for detecting 233 and 258 bp PCR products of the gene ldhA. The ubiquitin-protein ligase gene UBC6, with high transcriptional stability UBC6 (strain pTEF1-pctMe-VP16) was used as a reference gene for normalization of the expression levels.

3. Results

3.1. Regulation of ldhA expression and its effect on D-lactic acid production and PDLA accumulation

To control the D-lactic acid production in S. cerevisiae, the expression of stereospecific D-lactate dehydrogenase gene (ldhA) from L. mesenteroides (Baek et al., 2016) was adjusted with doxycycline controlled Tet-On method (Fig. 1) (Bellí et al., 1998; Gossen et al., 1995; T. Das et al., 2016). The system contained constitutively expressed pTF TetR-VP16 upstream of the core promoter derived from the ENO1 promoter (Rantasalo et al., 2018a), and the ldhA gene. Eight binding sites were chosen based on previous studies (Bellí et al., 1998; Rantasalo et al., 2016) to enable wide range of different expression levels from low to high in the tunable Tet-On expression system. The binding of TetR-VP16 to the binding sites was controlled with the concentration of the docycline in the media. The endogenous D-lactic dehydrogenase gene (DLD1) was deleted to prevent the oxidation of produced D-lactic acid and thus to increase the D-lactic availability for polymerization. The strain was further engineered by integration of propionyl-CoA transferase gene pctMe from M. elsdenii with constitutive pTEF1 promoter (strain pTEF1-pctMe-Tet-On-ldhA) and by integration of an engineered PHA synthase gene phaC1Pre from Pseudomonas resinovorans carrying four amino acid substitutions, E130D, S325T, S477G, and Q481K, with constitutive pTDH3 promoter (strain phaC1Pre_1x).

The PDLA and (LA-3HB) pathways are presented in Supplemental Fig. S2. The strains expressing only tetR-VP16, pctMe, or ldhA (Table 2) were used as controls. The constructed strains were first grown with different doxycycline concentrations in the Bioscreen C instrument in multiwell plates for 15 h. The expression of pctMe or tetR-VP16 under constitutive promoters did not affect cell growth (Supplemental Table S1, Supplemental Fig. S3). The highest docycline level of 10 mg L\(^{-1}\) had only very minor effect on the specific growth rate and the final OD\(_{600}\) of the control strains pTEF1-pctMe or the parent strain CEN. PK111-9A. The expression of the ldhA gene from the TDH3 promoter or from the Tet-On construct with 10 mg L\(^{-1}\) docycline decreased the growth rate by approximately 50% in comparison to the parent strain CEN.PK111-9A.

To measure the D-lactic acid and the PDLA production at the different expression levels of the ldhA, the PDLA strain (strain phaC1Pre_1x) and the corresponding control strain without PHA synthase (strain pTEF1-pctMe-Tet-On-ldhA) were grown with 20 mg L\(^{-1}\) of glucose and the PDLA synthase activity from 5 to 10 g L\(^{-1}\). Decreasing the docycline concentration from 0.5 to 0.1 mg L\(^{-1}\) by decreasing the doxycycline concentration from 7.5 to 0 mg L\(^{-1}\) (Fig. 1E, Supplemental Fig. S5). The ldhA transcription was downregulated accordingly when doxycycline was decreased (Fig. 1G). The accumulation of intracellular PDLA correlated linearly with the production of extracellular D-lactic acid (Fig. 2). The time profile of intracellular PDLA accumulation was followed with strain phaC1Pre_1x grown with 1, 3, and 5 mg L\(^{-1}\) doxycycline. The majority of the PDLA polymer accumulated already within the first 24 h (Fig. 1H). Only small 13% increase in PDLA was observed from 24 h to 72 h with 1 and 5 mg L\(^{-1}\) doxycycline concentrations. The maximum 47% PDLA percentage of CDW was obtained at 72 h with 5 mg L\(^{-1}\) doxycycline (Fig. 1F). The PDLA production was also calculated as mg L\(^{-1}\) -1. Result showed that from 24 h to 72 h the PDLA titer increased only 5% and 15% with 3 mg L\(^{-1}\) and 5 mg L\(^{-1}\) doxycycline, respectively (Supplemental Fig. S6).

The mRNA levels between the constitutive pTDH3 promoter and doxycycline-controlled Tet-On system were also compared. The ldhA expression from the constitutive pTDH3 promoter was measured from the control strain pTDH3-ldhA and from the strain used in our previous study pTDH3-ldhA-2u-pTDH3_phaC1Pre (H5520) (Ylinen et al., 2021). Their expression was on average 24% lower than expression from the constitutive promoter and 44% higher compared to the Tet-On promoter with 7.5 mg L\(^{-1}\) doxycycline.

3.2. Comparison of the two PHA synthases and their expression levels for PDLA production

The PHA synthases phaC1Pre from P. resinovorans (Yang et al., 2011) and phaC1437sp6-16 from Pseudomonas sp. MBE 6–19 (Yang et al., 2010) were compared for their D-lactic acid polymerization efficiencies in S. cerevisiae. While both synthases carry the same amino acid substitutions E130D, S325T, S477G, and Q481K, their amino acid identity is
only approximately 80% (Yang et al., 2011). One, three, or six copies of each PHA synthase gene under the pTDH3 promoter were integrated into the control strain pTEF1-pctMe-TetOn-ldhA (Table 2). In cultivations of the resulting strains, the ldhA expression was adjusted with doxycycline concentration of 5 mg L\(^{-1}\) that resulted in the highest PDLA production in the previous experiment, and in addition with two higher concentrations of 6.0 and 7.5 mg L\(^{-1}\). Cells were grown with 20 g L\(^{-1}\) glucose for 48 h. When the PHA synthase copy number increased from one to three or six copies, most of the strains showed delay in growth, glucose consumption, production of acetate and D-lactic acid, and PDLA accumulation (Fig. 3, Supplemental Fig. S7). Only the strain with three copies of the PHA synthase gene phaC1Pre (strain phaC1Pre_3x) accumulated more PDLA than strain with one copy of the corresponding gene (phaC1Pre_1x), but this improvement can be explained

Fig. 1. A: Schematic presentation of the Tet-On method used in this study to control expression of the D-lactate dehydrogenase gene (ldhA) from L. mesenteroides. Increase in doxycycline concentration results in higher binding of the synthetic transcription factor and enhanced ldhA expression. B–H: The results of the cultivation of the PDLA strain phaC1Pre_1x and its control strain pTEF1-pctMe-TetOn-ldhA for 72 h with 20 g L\(^{-1}\) glucose and with doxycycline concentrations of 0, 1, 2, 3, 4, 5, 7.5, and 10 mg L\(^{-1}\). B, C: Growth as OD\(_{600}\), D, E: D-lactic acid production (g L\(^{-1}\)), F, H: PDLA accumulation as % of CDW (strain PhaC1Pre_1x). The values represent averages of three biological replicates. Individual data points are presented with triangles, squares, diamonds, or circles. G: The ldhA transcription relative to expression of the ubiquitin-protein ligase gene (UBC6) in the strain pTEF1-pctMe-TetOn-ldhA at 6 h, and the control strains pTDH3-ldhA-2u-pTDH3_phaC1Pre and pTDH3-ldhA at 16h. The circles represent average values of the three biological replicates obtained with two oligo pairs (Table 1).
by the higher D-lactic acid production of the strain phaC1Pre_3x (Supplemental Fig. S8D). The strain with one copy of PHA synthase gene phaC1437Ps6-19 (strain phaC1437_1x) accumulated the most PDLA of all strains studied, 5.6% of CDW, when ldhA expression was controlled with the highest doxycycline concentration, 7.5 mg L\(^{-1}\). This strain produced also the most extracellular D-lactic acid, 5.1 g L\(^{-1}\), and grew to the highest OD\(_{600}\) in 24 h, when compared to other strains grown with 7.5 mg L\(^{-1}\) doxycycline. Cultivation of this strain resulted to the lowest medium pH and highest ethanol and acetate production at 24 h (Supplemental Fig. S7). When the strain phaC1437_1x was controlled with lower doxycycline concentrations of 5.0 and 6.0 mg L\(^{-1}\), less PDLA accumulated, only 4.3% of CDW. The extracellular D-lactic acid formation and intracellular PDLA accumulation showed clear positive correlation also in this experiment with the strain expressing the PHA synthase phaC1437Ps6-19 (Supplemental Fig. S8).

### 3.3. Effect of the regulation of ldhA expression on the D-lactic acid content of the P(LA-3HB) copolymer

The Tet-On enabled regulation of the ldhA expression was exploited for controlling the D-lactic acid content in the copolymer P(LA-3HB). The 3-hydroxybutyryl-CoA pathway genes phaA and phaB1 from *C. necator* were integrated with the pTEF1 and pTDH3 promoters, respectively, into the PDLA strains phaC1Pre_3x and phaC1437_3x. These parental strains with three copies of the PHA synthase genes were respectively, into the PDLA strains phaC1Pre_3x and phaC1437_3x. The parental strains with three copies of the PHA synthase genes were respectively, into the PDLA strains phaC1Pre_3x and phaC1437_3x. These parental strains with three copies of the PHA synthase genes were respectively, into the PDLA strains phaC1Pre_3x and phaC1437_3x.

In this study we demonstrated that the D-lactic acid content of the P(LA-3HB) copolymer produced *in vivo* can be adjusted from 6 mol% to 93 mol% in the yeast *S. cerevisiae* by controlling expression of the lactate dehydrogenase encoding gene ldhA with a modified doxycycline dependent Tet-On expression system. Our results highlight the power of the controlled gene expression in tuning of the polymer composition and improving the overall polymer yield.

The ldhA expression correlated well with the doxycycline concentration, reaching the highest levels with the maximum 10 mg L\(^{-1}\) of doxycycline used. This expression level was approximately 30% higher than with the strong constitutive pTEF1 promoter showing the power of the modified Tet-On system combined with eight STF binding sites and the ENO1 core promoter. Also, the extracellular D-lactic acid concentration correlated with the increase in ldhA expression at doxycycline concentrations from 0 to 7.5 mg L\(^{-1}\). Above this doxycycline range, the extracellular D-lactic acid concentrations were similar indicating that possibly other factors such as the ldhA mRNA translation efficiency or the pyruvate availability limited the D-lactic acid formation. The amount of the produced extracellular D-lactic acid correlated positively with the D-lactic acid content of the copolymer P(LA-3HB) and with the total PDLA accumulation in the culture (Jung et al., 2010; Yang et al., 2010) or control of the oxygen availability (Goto et al., 2010; Yamada et al., 2009, 2010, 2011). In addition to this precise ldhA control, the Tet-On method showed an increase in the copolymer accumulation in

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**Fig. 2.** The correlation of the D-lactic acid concentration produced to the media (g L\(^{-1}\)) to the PDLA accumulation % of CDW in the strain phaC1Pre_1x, in which the ldhA expression was controlled with 1, 3, and 5 mg L\(^{-1}\) doxycycline (A–C) or with 0, 1, 2, 3, 4, 5, 7.5, and 10 mg L\(^{-1}\) doxycycline (D). The samples were analyzed at 24 h (A), 48 h (B), and at 72 h (C, D).

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**3.4. Molecular weights of the produced PDLA and P(LA-3HB) polymers**

In order to compare the molecular weights of the PDLA and P(LA-3HB) produced with the PHA synthases phaC1Pre and phaC1437Ps6-19, the strains phaC1Pre_1x, phaC1437_1x, phaC1Pre_3x, and phaC1437_3x were grown for 72 h with 20 g L\(^{-1}\) glucose and 6 mg L\(^{-1}\) doxycycline. The molecular weights of the extracted polymers from both PDLA strains and from the strain PhaC1Pre_3HB were very similar at 24 h, in the range of weight average molecular weight (Mw) 7.1–8.4 kDa and number average molecular weight (Mn) 5.6–6.4 kDa (Table 4, Supplemental Fig. S11). However, the Mw and Mn of the copolymer P(LA-3HB) produced by the strain phaC1437_3HB were approximately 60% and 40% higher than with the other copolymer strains. The narrow dispersity (D) below 1.4 indicates that variation in molecular weights within each sample was small. The growth and metabolite results of this experiment are presented in Supplemental Fig. S12.

### 4. Discussion

In this study we demonstrated that the D-lactic acid content of the P(LA-3HB) copolymer produced *in vivo* can be adjusted from 6 mol% to 93 mol% in the yeast *S. cerevisiae* by controlling expression of the lactate dehydrogenase encoding gene ldhA with a modified doxycycline dependent Tet-On expression system. Our results highlight the power of the controlled gene expression in tuning of the polymer composition and improving the overall polymer yield.

The ldhA expression correlated well with the doxycycline concentration, reaching the highest levels with the maximum 10 mg L\(^{-1}\) of doxycycline used. This expression level was approximately 30% higher than with the strong constitutive pTEF1 promoter showing the power of the modified Tet-On system combined with eight STF binding sites and the ENO1 core promoter. Also, the extracellular D-lactic acid concentration correlated with the increase in ldhA expression at doxycycline concentrations from 0 to 7.5 mg L\(^{-1}\). Above this doxycycline range, the extracellular D-lactic acid concentrations were similar indicating that possibly other factors such as the ldhA mRNA translation efficiency or the pyruvate availability limited the D-lactic acid formation. The amount of the produced extracellular D-lactic acid correlated positively with the D-lactic acid content of the copolymer P(LA-3HB) and with the total PDLA accumulation in the culture (Jung et al., 2010; Yang et al., 2010) or control of the oxygen availability (Goto et al., 2010; Yamada et al., 2009, 2010, 2011). In addition to this precise ldhA control, the Tet-On method showed an increase in the copolymer accumulation in
yeast *S. cerevisiae* when the D-lactic acid content of the polymer increased. This is an opposite result to the earlier *E. coli* studies where the increase in the D-lactic acid content of the P(LA-3HB) copolymer correlated with lower total copolymer accumulation levels. The strains without any doxycycline in the media produced small concentrations of D-lactic acid, leading to 6 mol% and 17 mol% D-lactic acid content in the P(LA-3HB) (Table 3). This background D-lactic acid production could be potentially decreased by lowering the expression of the TetR-VP16 as high expression of sTF has been reported to result in the leakage of Tet-On systems (Roney et al., 2016). This could possibly enable the production of copolymers with even wider range of different D-lactic acid contents, reaching to levels below 6 mol%.

We showed accumulation levels of 5.6% PDLA and 19% P(LA-3HB) of CDW, which are over two and five fold higher, respectively, than reported in the previous studies with yeast (Lajus et al., 2020; Ylinen et al., 2021). The P(LA-3HB) accumulation was increased in the cells from 5% to 19% by increasing the D-lactic acid production in vivo. This result highlights the importance of sufficient precursor availability in PHA production in yeast, which has been observed also when PHB homopolymer has been produced in *S. cerevisiae* (Carlson and Srienc, 2006; Kocharin et al., 2012, 2013; Kocharin and Nielsen, 2013). We also studied the effect of higher expression of genes encoding PHA synthases. However, we did not observe any significant increase in PHA polymer accumulation by increasing the copy number the PHA synthase genes. In addition, the PDLA and P(LA-3HB) producing strains polymerized only 1.8–2.2% and 3.3–5.2% of the available D-lactic acid, respectively, and rest of the produced D-lactic acid was exported from the cells (Supplemental Table S3). Further studies are required to confirm the effect of the acetyl-CoA availability and the activity of the propionyl-CoA transferases.

The PDLA and P(LA-3HB) polymerization in *S. cerevisiae* strains was studied with two PHA synthases phaC1437Ps6-19 and phaC1Pre originating from different *Pseudomonas* species, but carrying the same four amino acid substitutions, E130D, S325T, S477G, and Q481K. These synthases were chosen for comparison since they have shown efficient D-lactic acid polymerization in *E. coli* (Yang et al., 2011) and in our previous study in *S. cerevisiae* (Ylinen et al., 2021). However, the results from *E. coli* study suggest that these two enzymes might have different activities towards D-lactyl-CoA as expression of phaC1437Ps6-19 and phaC1Pre resulted in approximately 50 and 65 mol% LA proportions in P(LA-3HB), respectively (Yang et al., 2011). The two PHA synthases showed indeed differences in copolymer P(LA-3HB) formation also in this study in *S. cerevisiae*. The copolymer strain with phaC1437Ps6-19 produced approximately 50% longer copolymers (Mw 12.2 kDa) with 5 mol% lower D-lactic acid content (88 mol% LA), than strain with phaC1Pre (Mw 7.5 kDa, 93 mol% LA). This correlation between higher D-lactic acid content and lower molecular weight in P(LA-3HB) copolymer was also observed in other P(LA-3HB) studies (Jung et al., 2010; Yamada et al., 2011). A recent *in vitro* polymerization study, which was carried out with PHA synthase phaC1PsSTQK from *Pseudomonas* sp. 61-3, proposes, that the high Tg of PDLA homopolymers (60 °C) inhibits PDLA polymer elongation by a PHA synthase when the PDLA polymer reaches molecular weight of approximately 3 kDa (Matsumoto et al., 2018). Even though several *in vivo* studies demonstrated the production of

Fig. 3. Comparison of the strains with the engineered PHA synthases, phaC1Pre and phaC1437Ps6-19, and their expression from one, three, or six copies the corresponding genes. The *ldhA* expression was controlled with 5, 6, and 7.5 mg L\(^{-1}\) doxycycline. **A–C:** Growth as OD\(_{600}\), **D–F:** D-lactic acid production (g L\(^{-1}\)), **G–H:** PDLA accumulation as % of CDW. The values represent averages of three biological replicates. The individual data points are presented with circles, squares, and triangles.
The results from the experiment where the strains phaC1Pre_1x, phaC1437_1x, phaC1Pre_3HB, and phaC1437_3HB were grown with 6 mg L\(^{-1}\) doxycycline was controlled with the Tet-On system using either 0, 1, 2.5, 5, 6, or 7.5 mg L\(^{-1}\) doxycycline. Fig. 4. Comparison of the copolymer strains phaC1Pre_3HB and phaC1437_3HB. The expression of the ldhA was controlled with the Tet-On system using either 0, 1, 2.5, 5, 6, or 7.5 mg L\(^{-1}\) doxycycline. A–B: The cell growth as cell dry weight; C–D: Production of D-lactic acid (g L\(^{-1}\)). E–F: Accumulation % of the P(LA-3HB) of CDW at 48 h, bars represent fraction of each monomer of CDW (g g\(^{-1}\)). The values represent averages of two or three biological replicates. The individual data points are presented with circles, triangles, or squares.

**Fig. 4.** Comparison of the copolymer strains phaC1Pre_3HB and phaC1437_3HB. The expression of the ldhA was controlled with the Tet-On system using either 0, 1, 2.5, 5, 6, or 7.5 mg L\(^{-1}\) doxycycline. A–B: The cell growth as cell dry weight; C–D: Production of D-lactic acid (g L\(^{-1}\)). E–F: Accumulation % of the P(LA-3HB) of CDW at 48 h, bars represent fraction of each monomer of CDW (g g\(^{-1}\)). The values represent averages of two or three biological replicates. The individual data points are presented with circles, triangles, or squares.

### Table 3

Comparison of the copolymer strains phaC1Pre_3HB and phaC1437_3HB. The expression of ldhA was controlled with the Tet-On system using either 0, 1, 2.5, 5, 6, or 7.5 mg L\(^{-1}\) doxycycline. The results represent averages of two or three biological replicates. CDW: Cell dry weight.

| Strain       | Doxycycline concentration (mg L\(^{-1}\)) | Total polymer % of CDW | Polymer titer (mg L\(^{-1}\)) | Polymer yield (mg g\(^{-1}\) glucose) | Lactic acid mol % | Ethanol (g L\(^{-1}\)) | Lactic acid (g L\(^{-1}\)) | Acetate (g L\(^{-1}\)) | CDW (g L\(^{-1}\)) |
|--------------|------------------------------------------|------------------------|-------------------------------|---------------------------------------|------------------|-----------------------|-------------------------|-------------------------|---------------------|
| PhaC1Pre_3HB | 0                                        | 3.0                    | 56                            | 2.7                                   | 17%              | 9.4                   | 0.10                    | 1.0                     | 1.9                  |
|              | 1                                        | 3.5                    | 63                            | 3.0                                   | 29%              | 10.1                  | 0.37                    | 0.9                     | 1.7                  |
|              | 2.5                                      | 6.0                    | 85                            | 4.0                                   | 72%              | 8.7                   | 2.2                     | 0.8                     | 1.5                  |
|              | 5                                        | 11.5                   | 158                           | 7.5                                   | 90%              | 8.7                   | 3.5                     | 0.6                     | 1.2                  |
|              | 6                                        | 14.2                   | 168                           | 8.0                                   | 93%              | 8.9                   | 3.6                     | 0.6                     | 1.3                  |
|              | 7.5                                      | 10.4                   | 130                           | 6.2                                   | 88%              | 8.6                   | 3.3                     | 0.7                     | 1.3                  |
| PhaC1437_3HB | 0                                        | 5.0                    | 135                           | 6.4                                   | 6%               | 9.1                   | 0.04                    | 1.7                     | 2.7                  |
|              | 1                                        | 5.1                    | 136                           | 6.5                                   | 10%              | 8.7                   | 0.11                    | 1.7                     | 2.7                  |
|              | 2.5                                      | 6.2                    | 137                           | 6.5                                   | 50%              | 8.5                   | 1.8                     | 1.4                     | 2.2                  |
|              | 5                                        | 17.9                   | 216                           | 10.3                                  | 88%              | 7.9                   | 4.0                     | 0.8                     | 1.4                  |
|              | 6                                        | 19.0                   | 232                           | 11.0                                  | 88%              | 7.9                   | 4.3                     | 0.6                     | 1.3                  |
|              | 7.5                                      | 9.1                    | 168                           | 8.0                                   | 77%              | 7.1                   | 3.2                     | 1.0                     | 1.8                  |

**Table 4**

The results from the experiment where the strains phaC1Pre_1x, phaC1437_1x, phaC1Pre_3HB, and phaC1437_3HB were grown with 6 mg L\(^{-1}\) doxycycline for 72 h. The results represent averages of two or three biological replicates. LA: D-lactic acid; CDW: Cell dry weight; Mn: Number average molecular weight; Mw: Weight average molecular weight; D: Dispersity; Polymdispersity.

| Strain          | Polymer % of CDW | LA mol% of polymer | Polymer titer (mg L\(^{-1}\)) | Mn (kDa) | Mw (kDa) | D (kDa) |
|-----------------|------------------|--------------------|-------------------------------|----------|----------|---------|
|                |                  | 0 h                | 24 h                          | 72 h     |          |         |
| phaC1Pre_1x     | 0.03 ± 0.02      | 3.6 ± 0.16         | 4 ± 0.09                      | 100%     | 100%     | 100%    |
|                 | 0.02            | 4.0 ± 0.50         | 4.9 ± 0.45                    | 100%     | 100%     | 100%    |
| phaC1437_1x     | 1.6 ± 0.10       | 8.7 ± 0.30         | 11.6 ± 0.88                   | 13%      | 94%      | 91%     |
| phaC1Pre_3HB    | 3.9 ± 0.12       | 10.5 ± 0.32        | 15 ± 1.42                     | 7%       | 90%      | 88%     |
| phaC1437_3HB    | 6.0 ± 0.10       | 13.6 ± 0.36        | 16 ± 1.85                     | 6%       | 94%      | 91%     |

### 5. Conclusions

Sustainable production of biosynthetic polymers with tunable and novel properties will become increasingly important in the future. New ways for optimizing production levels of the biopolymers and controlling their copolymer compositions are needed. In this study we showed how the modified Tet-On method enables tunable control of expression of the lactate dehydrogenase encoding gene ldhA in the yeast *S. cerevisiae*, which leads to control of formation of the D-lactic acid monomer *in vivo*. This in turn enabled us to improve production of the homopolymer PDLA and the copolymer P(LA-3HB), and most importantly to adjust the D-lactic acid content in the copolymer P(LA-3HB) from 6 mol% to up to 93 mol%, as a linear response to the ldhA expression. The synthetic transcription factor of the Tet-On system responds to the doxycycline levels in the medium. Thus, it provides an easy way to examine the effects and optimal levels of gene expression that are needed for strain improvement and for desired copolymer structures. The system reduces the need for strain constructions such as creating separate strains with different promoter strengths. It can be applied also for other genes within the polymer synthesis pathways, not only for controlling D-lactic acid synthesis *in vivo*, as successfully demonstrated in this work.

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Appendix A. Supplementary data

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