Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via β-alanine

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ABSTRACT

Microbial fermentation of renewable feedstocks into plastic monomers can decrease our fossil dependence and reduce global CO_2 emissions. 3-Hydroxypropionic acid (3HP) is a potential chemical building block for sustainable production of superabsorbent polymers and acrylic plastics. With the objective of developing *Saccharomyces cerevisiae* as an efficient cell factory for high-level production of 3HP, we identified the β-alanine biosynthetic route as the most economically attractive according to the metabolic modeling. We engineered and optimized a synthetic pathway for *de novo* biosynthesis of β-alanine and its subsequent conversion into 3HP using a novel β-alanine-pyruvate aminotransferase discovered in *Bacillus cereus*. The final strain produced 3HP at a titer of 13.7 ± 0.3 g L^{-1} with a 0.14 ± 0.0 C-mol C-mol^{-1} yield on glucose in 80 h in controlled fed-batch fermentation in mineral medium at pH 5, and this work therefore lays the basis for developing a process for biological 3HP production.

1. Introduction

3-hydroxypropionic acid (3HP) is a platform chemical, which can be converted into acrylic acid, 1,3-propandiol, malonic acid, biodegradable polyesters, and other valuable chemicals. In 2011 the world annual production of acrylic acid was 5000 kMT and the market size was USD 11.5 billion ("Acrylic Acid Market – Global Industry Analysis 2012–2018", Research and Markets, July 2013). Acrylic acid-derived products include superabsorbent polymers used in baby diapers and incontinence products, plastics, coatings, adhesives, elastomers, and paints. Currently, acrylic acid is primarily made by catalytic oxidation of propene. Establishment of 3HP production from glucose or other renewable carbon sources would provide a biosustainable alternative to acrylic acid production from fossil resources.

Several methods for biological production of 3HP have been described. Bacterial or fungal hosts can be used, where fungal hosts have the advantage of tolerance to low pH (3HP has a pKa value of 4.5), therefore enabling a more economical process without the need for neutralization during fermentation and acidification on product recovery. An attractive fungal host is baker's yeast, *Saccharomyces cerevisiae*, which is already used in several industrial processes for production of chemicals such as first and second generation bioethanol, succinate, isobutanol, resveratrol, amorphadiene and others (Borodina and Nielsen, 2014; Hong and Nielsen, 2012; Tippmann et al., 2013). Moreover *S. cerevisiae* can be engineered for tolerance to high concentrations of 3HP, thus making a robust production strain (Kildegaard et al., 2014).

In biological systems, 3HP can be synthesized via at least four different intermediates: glycerol, lactate, malonyl-CoA or β-alanine (Kumar et al., 2013). *Glycerol* conversion to 3HP via glycerol dehydratase and aldehyde dehydrogenase by engineered *Escherichia coli* and native and engineered *Klebsiella* strains has been reported most extensively (Kwak et al., 2012; Rathnasingh et al., 2009; Luo et al., 2012; Luo et al., 2011; Patrick et al., 2005; Suthers and Cameron, 2005). The application of the glycerol route in *S. cervisiae* is challenging because glycerol dehydratase is dependent on co-factor vitamin B$_{12}$, which is not naturally synthesized by *S. cervisiae*. Expression of active enzymes for conversion of *lactate* into 3HP has been described (Gokarn et al., 2012). However, this route is thermodynamically unfavorable (Henry et al., 2010) and likely to result in a mixture of

lactate and 3HP. Another route utilizes *malonyl-CoA* as precursor, which can be reduced to 3HP via combined action of malonyl-CoA reductase (malonate semialdehyde-forming) and 3-hydroxypropionate dehydrogenase or 3-hydroxyisobutyrate dehydrogenase. Alternatively a bi-functional malonyl-CoA reductase can be utilized, for example from *Chloroflexus aurantiacus* (Gokarn et al., 2012; Hügler et al., 2002). Furthermore the host can be engineered for improved supply of precursor and redox co-factor as the bi-functional malonyl-CoA reductase requires NADPH (Hügler et al., 2002; Chen et al., 2014). Finally, the route where *β-alanine* is converted into malonic semialdehyde either by the action of β-alanine-pyruvate aminotransferase (BAPAT) or γ-aminobutyrate transaminase (GABT) has been reported in *E. coli* (Liao et al., 2007) and yeast (Jessen et al., 2012) respectively (Fig. 1a). Malonic semialdehyde is further reduced into 3HP by the action of 3-hydroxypropionate dehydrogenase or 3-hydroxyisobutyrate dehydrogenase. β-alanine is a precursor for the biosynthesis of pantothenic acid (vitamin B_5), which by itself is a component of coenzyme A and acyl carrier protein, co-factors required by a large number of enzymes. In bacteria, β-alanine is produced via decarboxylation of L-aspartate by aspartate-1-decarboxylase encoded by gene *panD*. No ortholog of *panD* was found in the genome of *S. cerevisiae* and it was shown that the only active route for β-alanine biosynthesis was via spermine (White et al., 2001). Spermine can be naturally synthesized in yeast during ornithine metabolism. As it was our intention to engineer a high-3HP-producer, it was apparent that the

biosynthesis of β-alanine precursor via spermine will most likely not be able to provide high flux and therefore it was preferred to engineer an alternative route for β-alanine biosynthesis from aspartate in yeast.

Hence, to engineer a *de novo* route for 3HP production in yeast via the β-alanine pathway it was essential to identify enzymes capable of converting β-alanine into 3HP and also to establish a synthetic pathway for efficient synthesis of β-alanine itself. Although a possibility of using BAPAT for 3HP production in yeast was mentioned, this has, to the best of our knowledge, not been experimentally demonstrated. Specifically, according to Liao et al. (2010), enzymes having BAPAT activity can be obtained from *Pseudomonas putida* or *Pseudomonas aeruginosa* (Liao et al., 2007). We, however, found that the BAPAT enzyme from *P. putida* was not active in *S. cerevisiae*.

The aim of our study was to establish a biosynthetic route for production of 3HP from glucose via β-alanine in yeast *S. cerevisiae* and to demonstrate that 3HP production at low pH was possible.

2. Results

2.1. Evaluation of β-alanine metabolic route using genome-scale modeling

We used the iTO977 genome-scale metabolic model of *S. cerevisiae* to evaluate metabolic capabilities of the two promising
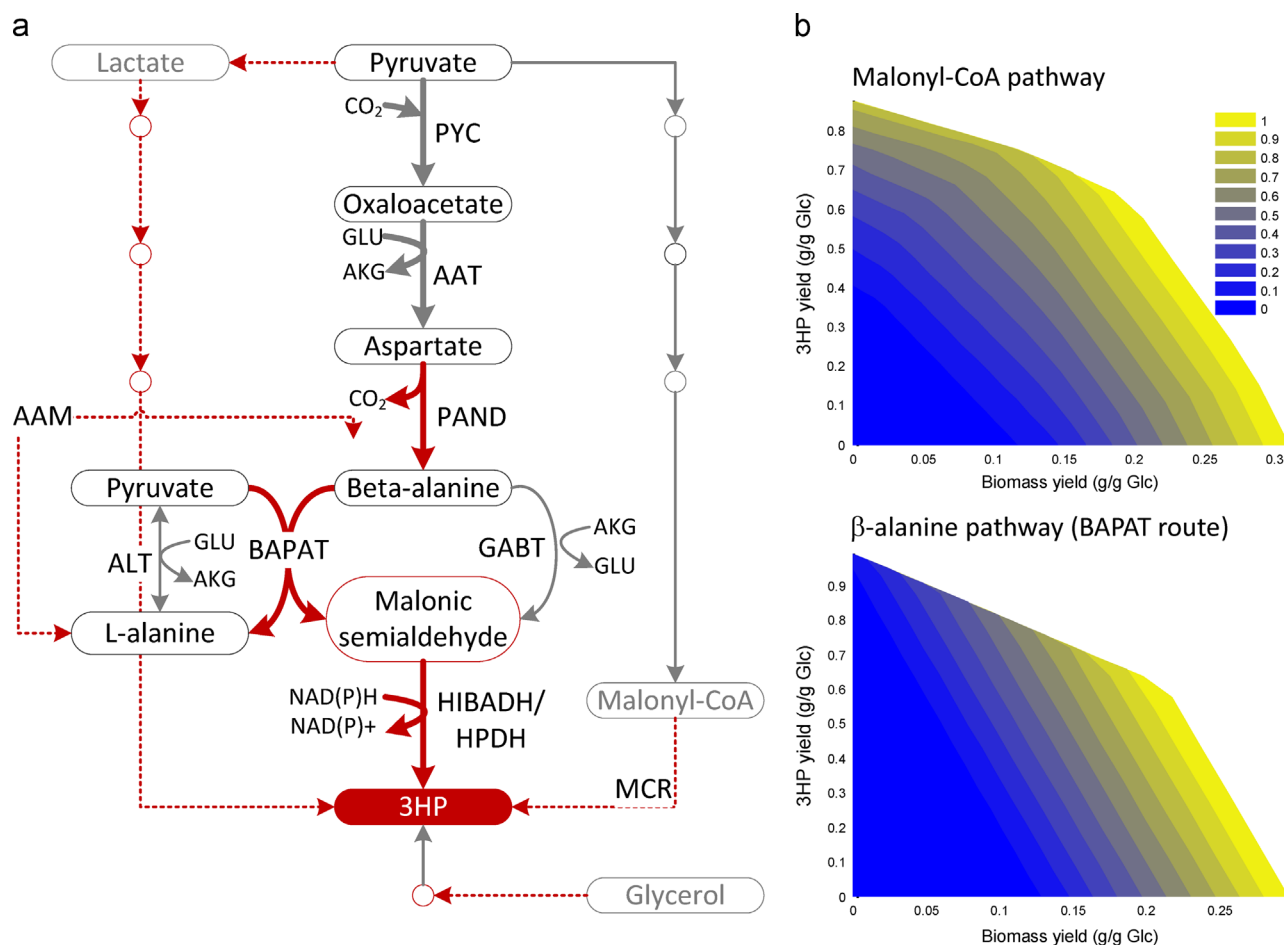
Fig. 1. *Biological routes towards 3-hydroxypropionic acid.* (a) Biosynthesis of 3HP can be achieved via glycerol, lactate, malonyl-CoA or β-alanine intermediates. The enzyme abbreviations are as follows: PYC – pyruvate carboxylase, AAT – aspartate aminotransferase, PAND – aspartate decarboxylase, BAPAT – β-alanine-pyruvate aminotransferase, GABT – γ-aminobutyrate transaminase, HIBADH – 3-hydroxyisobutyrate dehydrogenase, HPDH – 3-hydroxypropionate dehydrogenase, MCR – bi-functional malonyl-CoA reductase, AAM – 2,3-alanine aminomutase, ALT – alanine aminotransferase. Native reactions and intermediates are shown in gray, heterologous in red. The enzymes overexpressed in the described 3HP-producing strain are highlighted in bold. (b) Predicted maximum 3HP production limits as a function of aeration based on the iTO977 genome-scale metabolic model for the malonyl-CoA pathway and β-alanine pathway (BAPAT route). The color scale denotes oxygen uptake rate from 0 (blue) to maximal value set to 10 mmol g $DW^{-1} h^{-1}$ (yellow). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

routes towards 3HP, one via malonyl-CoA and another via β-alanine (Fig. 1b). We did not consider the glycerol route due to the difficulties associated with the expression of B$_{12}$-dependent glycerol dehydratase in *S. cerevisiae* or the lactate route because of the unfavorable thermodynamic constraints (Henry et al., 2010). The predicted maximum yield of the malonyl-CoA route is strongly oxygen-dependent because of the requirement of ATP for acetyl-CoA synthesis. The β-alanine route has a higher maximum theoretical yield, which is not significantly affected by the degree of aeration, and this makes the β-alanine route more attractive for an industrial process.

## 2.2. Identification of β-alanine-pyruvate aminotransferase (BAPAT) from B. cereus

3HP production in *E. coli* via a BAPAT enzyme from *P. aeruginosa* and *P. putida* has previously been described (Liao et al., 2007). We expressed *P. putida* BAPAT in combination with 3-hydroxypropionate dehydrogenases (HPDH) or 3-hydroxyisobutyrate dehydrogenases (HIBADH) in *S. cerevisiae*, but we did not observe conversion of β-alanine into 3HP by the recombinant cells. Therefore, we looked for an alternative BAPAT ortholog. The presence of an enzyme with BAPAT activity in the cell extracts of *B. cereus* has been reported previously

(Nakano et al., 1977). The enzyme was reported to have high substrate affinity with K_m of 1.1 mM for both L-alanine and pyruvate as compared to K_m 14 and 62 mM for L-alanine and pyruvate, respectively, in *Pseudomonas fluorescens* BAPAT. The gene encoding the BAPAT enzyme in *B. cereus* was, however, not known.

We therefore performed a sequence search with the BAPAT gene *aptA* from *Achromobacter dinitrificans* (GenBank: AY330220) against the genome sequence of *B. cereus* to discover an uncharacterized aminotransferase *yhxA* (GenBank: WP_002076750) as the closest homolog (35% identity). We built a 3D protein model based on the x-ray structure of *Bacillus anthracis* aminotransferase (PDB: 3N5M), which exhibits an overall sequence identity of 68% to YHXA and covers all but the first six residues (of 451). Eleven of the 14 amino acids of YHXA (S119, G120, S121, Y153, H154, E222, D255, V257, I258, K284, T320) located in the active site (defined as residues within 4 Å from pyridoxal-5'-phosphate) are conserved among BAPAT proteins with experimentally confirmed enzymatic activity and YHXA (Fig. 2b, Supplementary Results). Furthermore, W63 and R169 – both conserved and found in the active site, the latter being unique to BAPAT enzymes – are ideally positioned to form hydrogen bonds to the carboxyl group of the substrate (Fig. 2a).

The BAPAT-coding genes from *P. putida* and from *B. cereus* were synthesized in codon-optimized versions for *S. cerevisiae* and

a

b
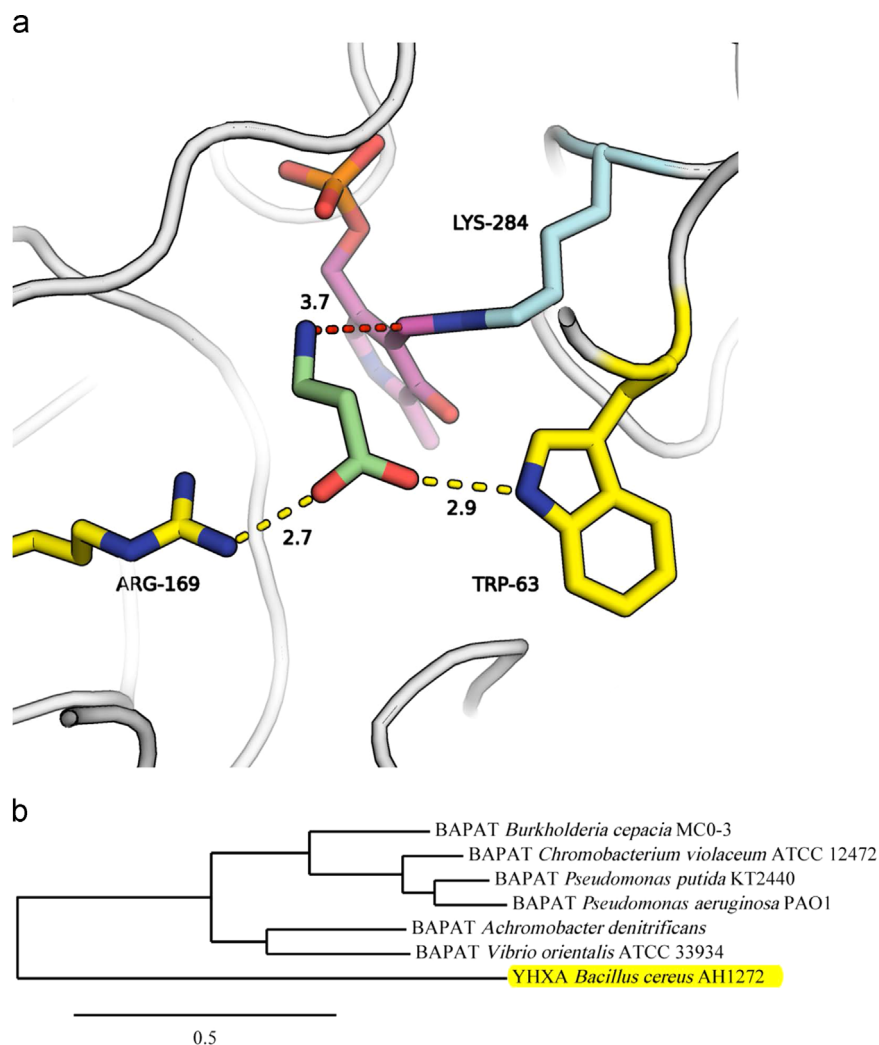
Fig. 2. *BAPAT structure and alignment.* (a) Predicted 3D structure of YHXA protein from *B. cereus* based on the structure of *B. anthracis* aminotransferase. The bound co-factor pyridoxal-5'-phosphate (PLP) is shown in purple, while K284 to which it is covalently linked is shown in light blue. The two proposed specificity-determining amino acid residues W63 and R169 are highlighted in yellow, β-alanine in green. Hydrogen bonds are indicated in yellow, the substrate amino group to PLP distance is indicated with red dashes. The figure was produced in PyMOL [The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC]. (b) Phylogenetic tree of β-alanine pyruvate aminotransferases with experimentally confirmed activity. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

expressed in *S. cerevisiae*. The cell extracts were analyzed for BAPAT activity using an enzymatic assay. BAPAT activity was detected in the cells expressing YHXA from *B. cereus*, but not in the cells expressing BAPAT gene from *P. putida* or in a control strain carrying an empty plasmid (Supplementary Fig. S1). No activity was observed when pyruvate in the substrate mix was substituted with α-ketoglutarate, confirming that the enzyme is β-alanine-pyruvate aminotransferase and not γ-aminobutyrate transaminase. Hence, we concluded that the identified gene indeed is the BAPAT gene from *B. cereus* and we chose to use this gene to establish 3HP production via β-alanine.

2.3. Reconstruction of the biosynthetic route from β-alanine to 3HP

To establish the conversion of β-alanine to 3HP, we expressed BAPAT from *B. cereus* or *P. putida* in *S. cerevisiae* in combination with four different 3-hydroxypropanoate dehydrogenases (HPDH) or four different 3-hydroxyisobutyrate dehydrogenases (HIBADH) enzymes (Fig. 3a). The tested HPDHs were from *Metallosphaera sedula* DSM 5348 (MsHPDH), *Sulfolobus tokadaii* str. 7 (StHPDH) or from *E. coli* str. K-12 (YDFG and RUTE) and HIBADHs were from *P. aeruginosa* (PaHIBADH), *P. putida* KT2440 (PpHIBADH), *B. cereus* ATCC14579 (BcHIBADH), or *Candida albicans* SC5314 (CaHIBADH). All the genes except for the *E. coli* genes were codon-optimized for expression in *S. cerevisiae*. To compare the efficiency of this route to the native γ-aminobutyrate transaminase route in *S. cerevisiae*, we also overexpressed the native (*UGA*1) or heterologous *gabT* genes (source: *Clostridium acetobutylicum* ATCC 824) in combination with the above mentioned HPDHs and HIBADHs. The yeast cells were cultured on a medium containing β-alanine and the concentration of 3HP was estimated by enzymatic assay (Fig. 3b). 3HP production from β-alanine was observed when *B. cereus* aminotransferase YHXA or *S. cerevisiae* γ-aminobutyrate transaminase UGA1 were combined with 3-hydroxyisobutyrate dehydrogenases or 3-hydroxypropanoate dehydrogenases. The best enzyme combination under the conditions tested was *B. cereus* aminotransferase YHXA (BcBAPAT) and *E. coli* 3-hydroxypropanoate dehydrogenase YDFG (EcYDFG), where 2.14 ± 0.09 g L^{-1} 3HP was obtained on the medium supplemented with 10 g L^{-1} β-alanine. No 3HP production was found when *P. putida* β-alanine-pyruvate aminotransferase or *C. acetobutylicum* γ-aminobutyrate transaminase was used in combination with 3-hydroxybutyrate dehydrogenases or 3-hydroxypropanoate dehydrogenases. For the conversion of malonic semialdehyde to 3HP, the majority of the NADPH-dependent enzymes (HPDH) performed better than the NADH-dependent enzymes (HIBADH). This could be due to the predominance of the reduced (NADPH) versus oxidized (NADP) co-factor forms and the opposite relationship for NADH and NAD in the cytosol of *S. cerevisiae* (Watanabe et al., 2007).

The identity of 3HP in the best sample was confirmed by an NMR analysis (Supplementary Fig. S2). The concentration measured by NMR was 1.75 ± 0.15 g L^{-1}, which was slightly lower than the value found by the enzymatic assay. The enzymatic assay likely leads to overestimation of 3HP concentration due to interference of other metabolites from the fermentation broth with the enzymatic reaction. Therefore, in all the following experiments the concentration of 3HP was measured by HPLC instead of enzymatic assay. In the HPLC analysis 3HP was well separated from the other components of the fermentation broth and quantification was therefore more accurate.

2.4. Reconstruction of biosynthetic route for β-alanine production from glucose via L-aspartate

The *S. cerevisiae* strains described above produced 3HP only when β-alanine was added to the medium. Cultivating the strains on glucose as the only carbon source or on a glucose medium supplemented with L-aspartate or L-alanine did not result in the 3HP formation (data not

shown) as could be anticipated from the previous report on the lack of β-alanine synthesis in *S. cerevisiae* during growth on glucose (White et al., 2001). In bacteria, β-alanine can be synthesized through decarboxylation of L-aspartate by aspartate-1-decarboxylase. Some glutamate decarboxylases can also have an activity towards L-aspartate (Wu et al., 1973). We expressed three different aspartate-1-decarboxylases, all with confirmed enzymatic activity in their native hosts, from *Corynebacterium glutamicum*, *Tribolium castaneum*, *E. coli*, and three glutamate decarboxylases from *Rattus norvegicus*, *E. coli*, and native *S. cerevisiae* GAD1 in combination with two enzymes converting β-alanine into 3HP (*B. cereus* BAPAT and *E. coli* YDFG). The resulting strains were evaluated for 3HP production on defined mineral medium containing glucose as the only carbon source or glucose and L-aspartate, and on feed-in-time medium for *S. cerevisiae* to simulate production in a fed-batch process. Only the *S. cerevisiae* strains expressing aspartate-1-decarboxylases from red flour beetle *T. castaneum* or from *C. glutamicum* were capable of producing 3HP (Fig. 3c). The 3HP titers for the strain overexpressing TcPAND, BcBAPAT and EcYDFG were 0.83 ± 0.08 g L^{-1} and 3.66 ± 0.72 g L^{-1} on mineral and feed-in-time media, respectively.

2.5. Increasing the flux towards 3HP via optimization of gene expression and improving supply of L-aspartate

Once the biosynthesis of 3HP from glucose via β-alanine had been established, the next goal was to improve the expression of the biosynthetic genes and to increase the flux towards aspartate. As this would require stable simultaneous overexpression of several genes, we used the previously developed integrative vector set EasyClone (Jensen et al., 2014). We tested the effect of overexpressing genes encoding native cytoplasmic aspartate aminotransferase *AAT2*, pyruvate carboxylases *PYC1* and *PYC2*, and combinations thereof. We also investigated the effect of multiple integration of the key biosynthetic genes, leading from aspartate to 3HP, using the vector designed for integration into retrotransposon TY4 elements (Supplementary Results). Increasing the copy number of integrated *BcBAPAT/EcYDFG* or of *TcPAND* genes led to improvement of 3HP titer in all the four background strains tested (reference, overexpressing *AAT2*, overexpressing *PYC1* & *PYC2* and overexpressing *AAT2* & *PYC1* & *PYC2*) (Fig. 4). The effect of multiple integrations of *TcPAND* was larger than that of multiple copies of *BcBAPAT/EcYDFG*. We also cloned all three genes (*BcBAPAT*, *EcYDFG* and *TcPAND*) into TY-vector and attempted to integrate all the genes in multiple copies. The resulting transformants did not secrete 3HP and colony PCR revealed that the strains lacked one or several of the *BcBAPAT*, *EcYDFG* or *TcPAND* genes. While integration into chromosomes is usually rather stable, the loss of the integrated genes by homologous recombination still can occur, and in this case there must have been a strong selection pressure for these events.

The increased expression of *AAT2*, *PYC1* and *PYC2* had a positive effect on 3HP production mainly in the strain with multiple copies of *TcPAND* gene, thus indicating that the main flux control resided with *TcPAND*, when it was in single copy, however once *PAND* activity was increased, the flux control partially moved to the aspartate biosynthesis enzymes. The positive effect of overexpressing pyruvate carboxylase genes was only observed on feed-in-time medium, which simulates fed-batch conditions, but not in the batch cultivation. This may be because under batch conditions, i.e., during growth on glucose and then on ethanol, there is high pyruvate carboxylase activity in the cells (Brewster et al., 1994) and therefore expression of additional copies of *PYC1/PYC2* did not have an effect. The highest titers were obtained for the strain SCE-R2-200 overexpressing *AAT2*, *PYC1*, *PYC2*, *BcBAPAT*, *EcYDFG*, and multiple copies of *TcPAND*: 1.27 ± 0.28 g L^{-1} and 8.51 ± 1.05 g L^{-1} on mineral and feed-in-time media correspondingly.
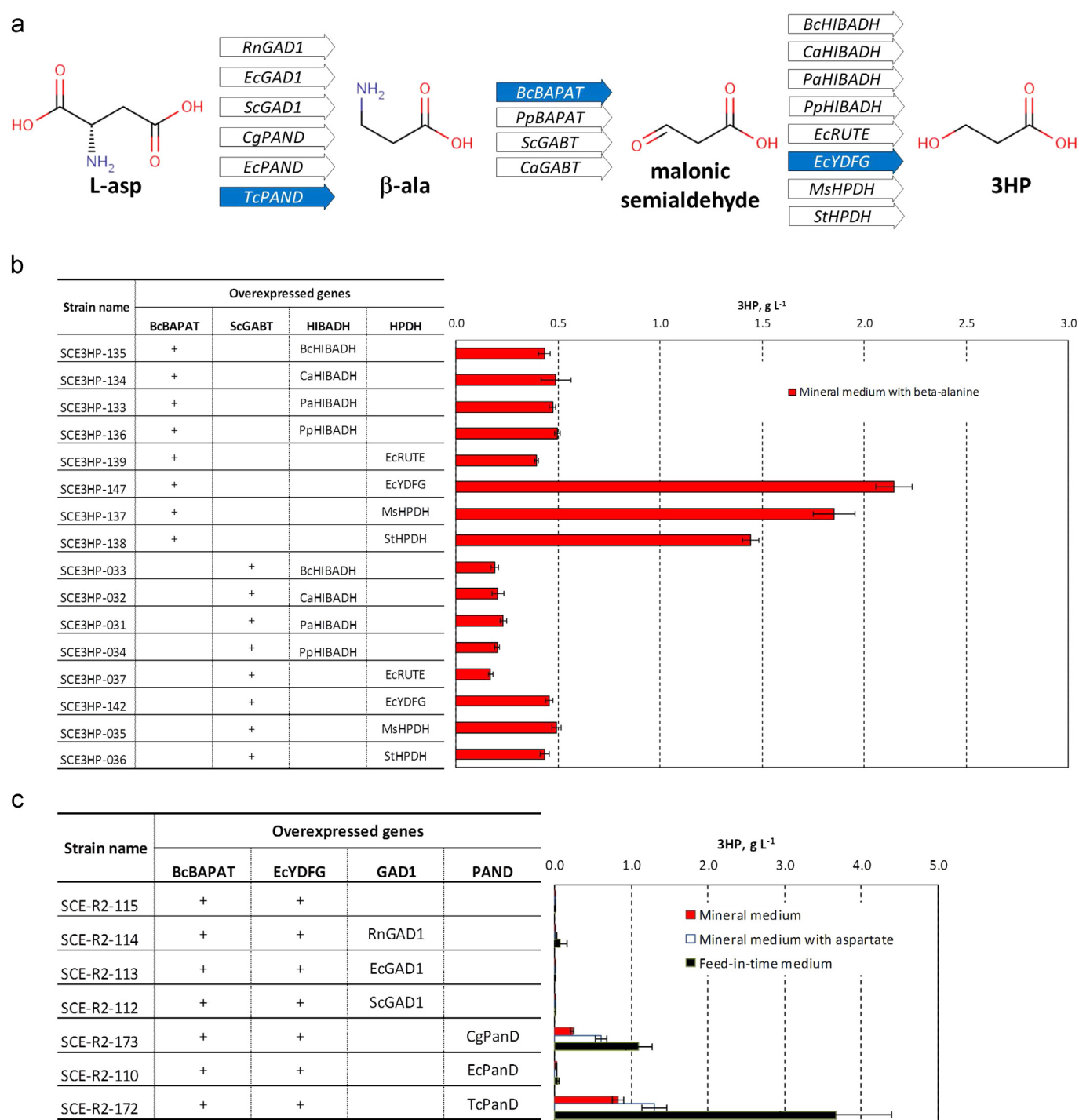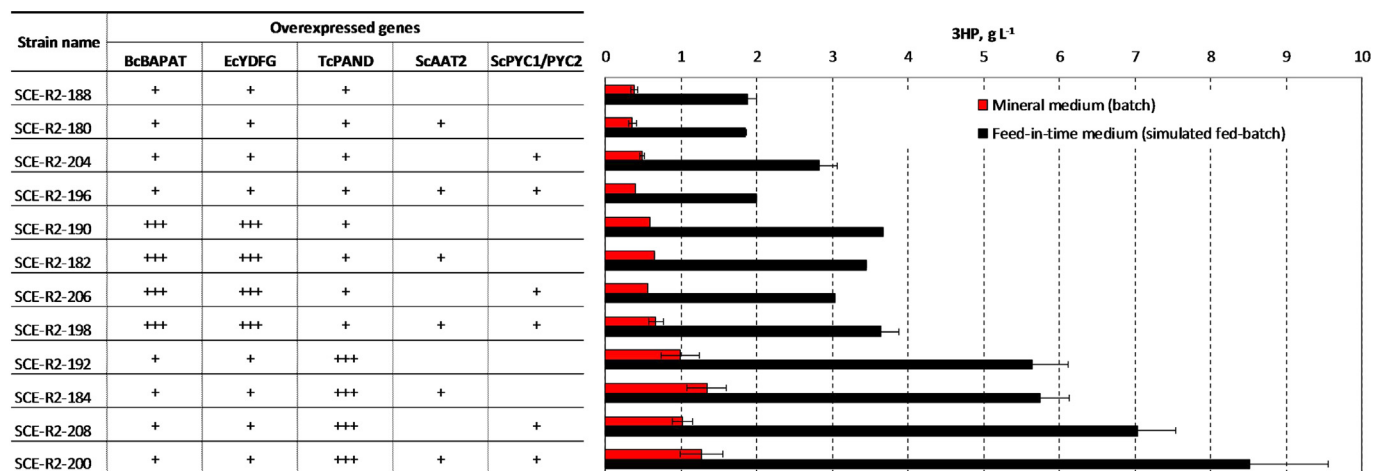
Fig. 3. *Production of 3HP by recombinant S. cerevisiae strains.* (a) Overview of the genes that were tested for reconstruction of *de novo* 3HP biosynthesis pathway in *S. cerevisiae*. The gene variants chosen for the final producing strain are highlighted in blue. (b) Conversion of β-alanine to 3HP by recombinant *S. cerevisiae* strains. Extracellular concentrations of 3HP in the fermentation broth of cells cultivated on mineral medium supplemented with $10 \, \text{g L}^{-1}$ of β-alanine as measured by enzymatic assay. The strains express different combinations of *HIBADH/HPDH* and *BAPAT/GABT* genes. (c) Production of 3HP from glucose by *S. cerevisiae* expressing *BcBAPAT* and *EcYDFG* genes in combination with *GAD1* or *PAND* variants measured by HPLC. The numbers are average values $\pm$ standard deviation for at least three individual strain isolates. "+" denotes that the gene was expressed in the given strain (the heterologous genes were expressed using 2 μ-based plasmids). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

2.6. Recycling of L-alanine

The BAPAT activity results in the formation of one L-alanine molecule per each β-alanine molecule formed (Fig. 1a). The generated L-alanine can either be recycled to pyruvate via various transamination reactions or via alanine aminotransferase, which transfers amino group to α-ketoglutarate to form glutamate. It was

also proposed that L-alanine can be converted to β-alanine via the action of a non-natural 2,3-alanine aminomutase (AAM) developed by directed evolution of L-lysine 2,3-aminomutase from *Bacillus subtilis* (Liao et al., 2010). The *S. cerevisiae* genome contains two genes encoding alanine aminotransferase enzymes, *ALT1* and *ALT2*, of which the latter is catalytically inactive (Peñalosa-Ruiz et al., 2012). We tested the effect of *ALT1* or *BsAAM* overexpression

Strain name	Overexpressed genes				
	BcBAPAT	EcYDFG	TcPAND	ScAAT2	ScPYC1/PYC2
SCE-R2-188	+	+	+		
SCE-R2-180	+	+	+	+	
SCE-R2-204	+	+	+		+
SCE-R2-196	+	+	+	+	+
SCE-R2-190	+++	+++	+		
SCE-R2-182	+++	+++	+	+	
SCE-R2-206	+++	+++	+		+
SCE-R2-198	+++	+++	+	+	+
SCE-R2-192	+	+	+++		
SCE-R2-184	+	+	+++		
SCE-R2-208	+	+	+++		+
SCE-R2-200	+	+	+++	+	+

Fig. 4. *Strain optimization.* Influence of integrating multiple copies of genes and of overexpression of precursor supply genes on 3HP titer in mineral and feed-in-time media. The concentration of 3HP in the culture broth was determined by HPLC. "+" denotes that a single copy of gene was integrated into the genome, "++" denotes that multiple copies of gene were integrated into the genome. The numbers are average values ± standard deviation for at least three individual strain isolates.

on 3HP production. For the test a leucine-negative *S. cerevisiae* strain was constructed, which overexpressed *PYC1*, *PYC2*, *BcBAPAT*, *EcYDFG*, and multiple copies of *TcPAND*. Multiple transformants were screened for 3HP production and the best performing strain (SCE-R3-46) was selected. It was further transformed with either an empty integrative vector to complement the leucine auxotrophy or with vectors designed for overexpression of *BsAAM* or *ALT1*. Overexpression of either *BsAAM* or of *ALT1* genes decreased the 3HP titer on feed-in-time medium by correspondingly 18% or 10% (Supplementary Fig. S3).

2.7. Production of 3-hydroxypropionic acid in controlled fed-batch fermentation

Strain SCE-R2-200 described above was cultivated in aerobic fed-batch reactor with glucose-limited feed at pH 5 in triplicates. The medium contained mineral salts, vitamins and glucose as the sole carbon source. The strain produced 3-hydroxypropionic acid at 13.7 ± 0.3 g L^{-1} titer, $14 \pm 0\%$ C-mol C-mol^{-1} yield on glucose and an overall process productivity of 0.17 ± 0.0 g L^{-1} h^{-1}. No significant amounts of by-products such as acetate, ethanol or glycerol were detected at the end of the fermentation (Fig. 5). Production of 3HP at low pH was also evaluated. Using a simplified constant feed protocol, 12.2 g L^{-1} and 9.2 g L^{-1} of 3HP were obtained when pH was maintained at correspondingly 5 and 3.5 throughout the fermentation (Supplementary Fig. S4).

3. Discussion

In production of organic acids, it is advantageous to carry out the fermentation process at low pH: it saves the costs of neutralization and subsequent acidification to obtain the free acid and also reduces the likelihood of bacterial contamination. Therefore fungal hosts are often preferred (e.g., *Aspergillus niger* for citric acid production and yeast for lactic acid and succinic acid production).

We engineered a heterologous route for *de novo* production of 3-hydroxypropionic acid via β-alanine in yeast *S. cerevisiae*. To the best of our knowledge, this is the first report on utilization of β-alanine-pyruvate aminotransferase for 3HP production in yeast. Overexpression of BAPAT from *B. cereus* resulted in higher 3HP titers than overexpression of the native γ-aminobutyrate transaminase, presumably due to higher intracellular concentration of pyruvate than α-ketoglutarate, which are the substrates for BAPAT and GABT, respectively. Interestingly, while the uncharacterized
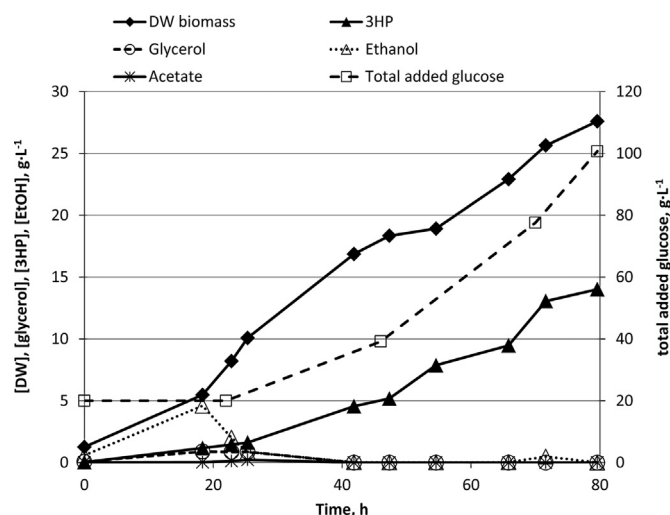
Fig. 5. *Fed-batch cultivation of 3HP producing strain.* Growth and metabolite production profiles in glucose-limited controlled fed-batch cultivation of strain SCE-R2-200 at pH 5. The cultivation was performed in triplicates, here a representative graph is shown.

aminotransferase YHXA from *B. cereus* has a very low sequence identity to the other known BAPAT enzymes, the residues in the active site within 4 Å of the pyridoxal-5′-phosphate moiety are strongly conserved between the known BAPAT enzymes. Furthermore, R169 is ideally positioned to hold the carboxyl group of the β-alanine substrate in collaboration with W63 on the opposite side of the substrate-binding pocket. This arginine residue not only appears to be conserved among all characterized BAPAT enzymes, it is also unique to them and is possibly involved in defining the specificity of BAPAT enzymes.

3HP is a source of commodity chemicals and needs to be produced at a price competitive to the petrochemical acrylic acid. Despite the promising production parameters (~14 g L^{-1} titer and 14% C-mol/C-mol glucose yield), the titer and the yield need to be improved at least 4–5-fold to reach commercial feasibility for production of 3HP from glucose, while volumetric productivity must be increased more than 10-fold (Van Dien, 2013). With the maximal theoretical yield of nearly one gram 3HP per gram of glucose via β-alanine pathway (Fig. 1b), it should, however, be possible to achieve the commercial process requirements by further strain and process optimization, and we are therefore

confident that this work forms the basis for a commercial 3HP production using *S. cerevisiae*.

4. Conclusions

In this study we engineered a synthetic pathway for *de novo* biosynthesis of β-alanine and its subsequent conversion into 3HP in yeast *S. cerevisiae*. Furthermore, we optimized the expression of the critical enzymes in the pathway and increased aspartate biosynthesis to obtain a high-3HP-producing strain. We demonstrated 3HP production at low pH 3.5.

5. Methods

5.1. Computational methods

Genome-scale metabolic model iTO977 (Österlund et al., 2013) was obtained in a COBRA Toolbox compatible SBML format from http://www.sysbio.se/. The relevant reactions for 3HP production using either the malonyl-CoA or β-alanine route (BAPAT-dependent) were added to the model. The production envelopes for 3HP production at different aeration levels were generated using the COBRA Toolbox (Schellenberger et al., 2011) in Matlab (Mathworks, Inc., Natick, MA). The maximum glucose uptake rate for all the simulations was set to 10 mmol g $DW^{-1} h^{-1}$ and the oxygen uptake rates were varied from 0% to 100% of the maximum glucose uptake rate, i.e., from 0 to 10 mmol g $DW^{-1} h^{-1}$, in order to simulate different aeration levels. For the fully anaerobic simulation, free uptake of following compounds was allowed in the model to enable biomass production: 4,4-dimethylzymosterol, tetradecanoyl-9-ene acid, hexadecanoyl-9-ene acid, octadecanoyl-9-ene acid, ergosta-5,7,22,24(28)-tetraen-3-beta-ol, lanosterol, zymosterol.

5.2. Strains and chemicals

S. cerevisiae CEN.PK strains were obtained from Peter Kötter (Johann Wolfgang Goethe-University Frankfurt, Germany). Easy-Clone plasmids used in this work are described in Jensen et al. (2014) (Liao et al., 2010). The chemicals were from Sigma-Aldrich. The 3-hydroxypropionic acid 30% solution was purchased from TCI. Pfu TURBO DNA polymerase was from Agilent Technologies Inc.

5.3. Media

Synthetic complete medium as well as drop-out media and agar plates were prepared using pre-mixed drop-out powders from Sigma-Aldrich. Mineral medium was prepared as described previously (Jensen et al., 2014). Synthetic fed-batch medium for *S. cerevisiae* M-Sc.syn-1000 was purchased from M2P labs GmbH (Germany). The medium was supplemented with the supplied vitamins solution (final 1% v/v) and the enzyme mix (final concentration 0.5% v/v) immediately prior to use.

5.4. Metabolites analysis by HPLC

The extracellular metabolites concentrations were analyzed on HPLC. Therefore 25 μl of the sample was analyzed for 45 min using an Aminex HPX-87H ion exclusion column with a 1 mM H_2SO_4 flow of 0.6 ml/min. The temperature of the column was 60 °C. The refractive index at 45 °C and the UV absorption at 210 nm were measured. Glucose, glycerol and ethanol were detected using a RI-101 Refractive Index Detector (Dionex). 3HP, pyruvate, succinate and acetate were detected with a DAD-3000 Diode Array Detector

at 210 nm (Dionex). The UV spectrum was recorded with a diode array and the identity of the 3-hydroxypropionic acid was additionally verified by comparison with the spectrum of the standard.

5.5. NMR spectroscopy

The spectra were acquired on a Bruker AVANCE III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a four channel 5 mm inverse detection probe head with pulse-field gradients along the Z axis.

Spectra were run at 25 °C with presaturation of the water signal and a recycle delay of 6 s. For quantification purposes, formate was added as an internal concentration standard and spectra run with a repetition delay of 66 s.

5.6. Cultivation in microtiter plates

At least six single colonies originating from independent transformants were inoculated in 0.5 ml drop-out liquid medium without uracil, histidine, and leucine in a 96-deep well microtiter plate with air-penetrable lid (EnzyScreen, NL). The plates were incubated at 30 °C with 250 rpm agitation at 5 cm orbit cast overnight. 50 μl of the overnight cultures were used to inoculate 0.5 ml mineral medium or synthetic fed-batch medium in the 96-deep well plate. Fermentation was carried out for 72 h at the same conditions as above. For some experiments the mineral medium was supplemented with 10 g L^{-1} β-alanine.

At the end of the cultivation OD_{600} was measured as following: 10 μl of the sample was mixed with 190 μl water and absorbance was measured at 600 nm wavelength in a microplate reader BioTek Synergy MX (BioTek). The culture broth was spun down and the supernatant analyzed for 3-hydroxypropionic acid concentration using enzymatic assay or HPLC.

5.7. Controlled fermentations

SCE-R2-200 glycerol stock (0.3 ml) was inoculated in 150 ml mineral medium in a 500 ml baffled shake flask and propagated at 30 °C with 250 rpm agitation for about 24 h. The culture was concentrated down to 50 ml by centrifugation at $4000 \times g$ for 2 min and used to inoculate 0.5 L medium in 1 L-Sartorius reactor. The final medium in the reactors contained per liter: 15 g $(NH_4)_2SO_4$, 6 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 4 ml trace metals solution, 2 ml vitamins solution, 0.4 ml antifoam A (Sigma-Aldrich), and 44 g dextrose. The initial cultivation volume was 0.5 L. Dextrose was autoclaved separately. Vitamins solution was sterile filtered and added to the medium after autoclavation. The trace metal and vitamins solutions were the same as described previously (Jensen et al., 2014).

The agitation rate was 800 rpm, the temperature was 30 °C, aeration was 1 L min^{-1} air and pH was maintained at 5.0 by automatic addition of 2 N NaOH. Carbon dioxide concentration in the off-gas was monitored by an acoustic gas analyzer (model number 1311, Bruël & Kjær). Once the glucose was exhausted, which was observed from decline in CO_2 production and was also confirmed by residual glucose detection using glucose strips Glucose MQuant™ (Merck Millipore), the feed was started at 5 g h^{-1}. The feed contained per liter: 45 g $(NH_4)_2SO_4$, 18 g KH_2PO_4, 3 g $MgSO_4 \cdot 7H_2O$, 12 ml trace metals solution, 6 ml vitamins solution, 0.6 ml antifoam A, and 176 g dextrose. Dextrose was autoclaved separately, vitamins solution was sterile filtered and added to the feed after autoclavation. 24 h after the feed start the feed rate was ramped up to 10 g h^{-1} and 48 h after the feed start it was further increased to 15 g h^{-1}. The total feed volume used per reactor was 0.5 L. The measured glucose concentration did not

exceed $0.3 \, \text{g L}^{-1}$ during the fed-batch phase. The reactors were sampled twice a day to measure biomass dry weight and metabolites. For metabolites analysis the sample was immediately centrifuged and the supernatant was stored at $-20\,°C$ until HPLC analysis.

For estimating the effect of pH on 3HP production, the fermentations were carried out with a constant feed rate of $10 \, \text{g min}^{-1}$.

5.8. Modelling of YHXA

A homology model of YHXA was built using the HHpred server (http://toolkit.lmb.uni-muenchen.de/hhpred; PMID 15980461). The model was based on the x-ray structure of *B. anthracis* aminotransferase (3N5M), which exhibits 68% sequence identity to YHXA and covers residues 7–451 (of 451 residues; 98.4%). Following homology modeling, a pyridoxal-5′-phosphate (PLP) moiety was manually added from the structure of *Chromobacterium violaceum* ω-transaminase (4A6T; PMID 22268978) and linked to the side chain amino group of the universally conserved K284. The monomer YHXA model with the PLP co-factor was duplicated and arranged as a dimer by alignment to the template structure. Finally, the substrate β-alanine was manually docked into the binding site.

Author contributions

IB and JN conceived the study; IB, KKR, NJ, JM, SS, and FÖ performed the experiments and analyzed the data; THB performed the 3D protein modeling; PL carried out NMR analysis; MH performed metabolic modeling; IB drafted the manuscript and all the authors contributed to preparing the final version of the manuscript.

Competing financial interests statement

The authors declare no competing financial interests.

Significance statement

Sustainable production of chemicals from renewable feedstocks using engineered microorganisms is a way to reduce dependence on fossil resources and decrease carbon dioxide emissions. One of the attractive chemicals is 3-hydroxypropionic acid, which can become the basis for sustainable production of acrylic plastics and biodegradable polyesters. This is, to the best of our knowledge, the first report on high-level production of 3-hydroxypropionic acid from a sugar feedstock. We engineered and optimized a synthetic pathway for *de novo* biosynthesis of β-alanine and its subsequent conversion into 3HP. We employed yeast as the cell factory to enable a more economical low pH process.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.10.003.

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