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REVIEW ARTICLE

Malonyl-CoA pathway: a promising route for 3-hydroxypropionate biosynthesis

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ABSTRACT

3-Hydroxypropionate (3HP) is an attractive platform chemical, serving as a precursor to a variety of commodity chemicals like acrylate and acrylamide, as well as a monomer of a biodegradable plastic. To establish a sustainable way to produce these commercially important chemicals and materials, fermentative production of 3HP is widely investigated in recent years. It is reported that 3HP can be produced from several intermediates, such as glycerol, malonyl-CoA, and β-alanine. Among all these biosynthetic routes, the malonyl-CoA pathway has some distinct advantages, including a broad feedstock spectrum, thermodynamic feasibility, and redox neutrality. To date, this pathway has been successfully constructed in various species including *Escherichia coli*, yeast and cyanobacteria, and optimized through carbon flux redirection, enzyme screening and engineering, and an increasing supply of energy and cofactors, resulting in significantly enhanced 3HP titer up to 40 g/L. These results show the feasibility of commercial manufacturing of 3HP and its derivatives in the future.

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Introduction

3-Hydroxypropionate (3HP) is one of the top 12 value-added chemicals from biomass released by the US Department of Energy [1]. It naturally exists in a small number of thermophile archaea and bacteria, and participates in autotrophic carbon fixation cycles [2–5]. 3HP is a precursor in synthesis of various compounds, including 1,3-propanediol, acrylic acid, β-propiolactone and biodegradable polyhydroxyalkanoates containing 3HP monomer [6–10]. However, commercial application of 3HP was restrained due to its high production cost [11,12]. In recent years, much effort has been made to produce 3HP from an inexpensive renewable feedstock in a metabolic engineered microorganism [12–14].

There are three 3HP-producing pathways reported which are as shown in Figure 1. Although the glycerol pathway achieves the highest 3HP titer, it still has little market competitiveness because of an exogenous supply of coenzyme B12 [15,16], which is required for the activity of glycerol dehydratase [17]. Furthermore, the reducing power imbalance was also a burden for cellular metabolism [17]. In both β-alanine and malonyl-CoA pathways, 3HP is derived from common microbial metabolic intermediate, so that various sugars from lignocellulosic biomass can be used as a raw material for 3HP production. Additionally, the production of 3HP from glucose is redox neutral. Unfortunately, all recombinant strains harboring the β-alanine pathway presents a low 3HP yield, and the highest 3HP titer was 13.7 mg/L [18]. In contrast, the 3HP production *via* malonyl-CoA pathway has been dramatically improved in the past few years [19,20].

The malonyl-CoA pathway has been successfully reconstructed in *Escherichia coli* [15], *Saccharomyces cerevisiae* [21], and cyanobacteria [22]. It employs acetyl-CoA carboxylase (ACC) for the conversion of acetyl-CoA into malonyl-CoA, which is converted into 3HP with a two-step reduction catalyzed by malonyl-CoA reductase (MCR) (Figure 2). The genes encoding ACC widely reside in various organisms and participate in the synthesis of fatty acids [23,24], and the gene encoding MCR is derived from autotrophic thermophile *Chloroflexus aurantiacus* as part of its 3HP cycle for carbon fixation [3,25–27].

In order to improve 3HP production, malonyl-CoA pathway was optimized using the following four strategies: (1) redirecting carbon flux toward 3HP

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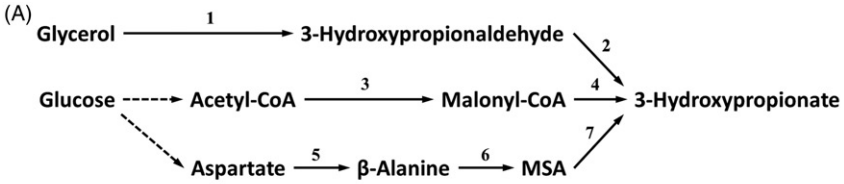

(B)

3HP biosynthetic pathway	Carbon source	Steps†	Coenzyme required	NAD(P)H yield (mol/mol 3HP)	ATP yield (mol/mol 3HP)	Highest 3HP titer reported (g/L)
Glycerol pathway	Glycerol	2	CoB12‡	1	0	83.8
Malonyl-CoA pathway	Glucose	2	Biotin	0	0	40.6
β-Alanine pathway	Glucose	3	-	0	0	13.7

† Steps, steps from carbon sources or central metabolites.
‡ CoB12, coenzyme B12.

Figure 1. Three pathways for 3-hydroxypropionate production. (A) Different metabolic routes for 3HP biosynthesis from glycerol or glucose. The enzymes involved are as follows: 1, glycerol dehydratase; 2, aldehyde dehydrogenase; 3, acetyl-CoA carboxylase; 4, malonyl-CoA reductase; 5, aspartate-1-decarboxylase; 6, β-alanine aminotransferase; 7, malonate semialdehyde reductase. (B) Comparison of those pathways for 3HP biosynthesis. MSA: malonate semialdehyde.

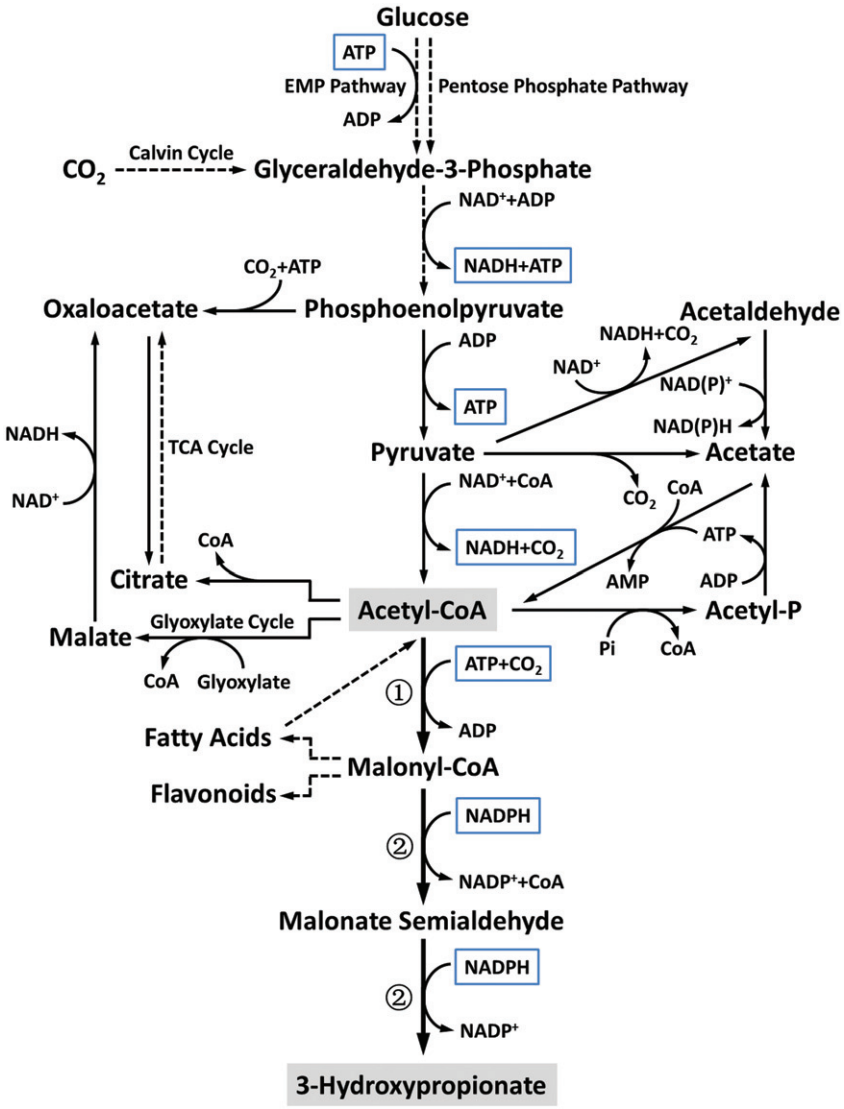

Figure 2. 3-Hydroxypropionate biosynthesis from glucose or CO_2 *via* malonyl-CoA pathway in recombinant microorganisms. The acetyl-CoA carboxylase (ACC, ①) is responsible for formation of malonyl-CoA from acetyl-CoA, and the malonyl-CoA reductase from *C. aurantiacus* (MCR, ②) catalyzes the two-step reduction of malonyl-CoA with NADPH to 3HP.

biosynthesis, (2) improving the catalysis of key enzymes, (3) enhancing cofactor and energy supply, and (4) selecting specific host strain.

Redirecting carbon flux toward 3HP biosynthesis

In metabolic engineering, redirecting carbon flux to the desired product is considered as an effective approach. In the malonyl-CoA pathway, acetyl-CoA and malonyl-CoA are two core intermediates, and are also involved in the formation of other metabolites (Figure 2). To enhance 3HP yield, a common approach is to manipulate the competing pathways to channel carbon flux to 3HP biosynthesis [28].

Redirecting carbon flux from pyruvate to malonyl-CoA

Under aerobic conditions, generation of acetyl-CoA from pyruvate is mainly catalyzed by the pyruvate dehydrogenase (PDH) complex in microbes. As a central metabolite, acetyl-CoA is shunted to multiple metabolic pathways such as the TCA cycle, acetate metabolism, glyoxylate cycle and malonyl-CoA formation (Figure 2) [29].

In *E. coli*, acetate metabolism is the second largest way of ATP supply after oxidative phosphorylation [30]. Therefore, acetate is commonly regarded as one of the representative byproducts [31]. There are two particular routes of acetate production from pyruvate in *E. coli* (Figure 2): in the phosphate acetyltransferase/acetate kinase (PTA-ACK) route, acetyl-CoA produced from pyruvate is further converted into acetate with acetyl-P as an intermediate. This route is initiated in the logarithmic phase to regulate the carbon flux balance and generate ATP for cell growth. In the pyruvate oxidase (POX) route, pyruvate is decarboxylated into acetate. This route probably plays a role in the late logarithmic phase and the stationary phase to maintain the balance of free CoA metabolic pool [32]. The 3HP production should be enhanced by the knockout of genes related with the above pathways. However, blocking the PTA-ACK route hardly showed any effect on the 3HP titer in the recombinant *E. coli* strain [15]. In accordance with this result, the production of an ester from acetyl-CoA remained largely unchanged, even though both PTA-ACK and POX pathways were deleted [33].

Actually, acetate reuse has been proved to be an effective strategy on reducing carbon loss during fermentation. Overexpression of acetyl-CoA synthetase (ACS), which regenerates acetyl-CoA from acetate, helps to channel carbon flux to acetyl-CoA and improve 3HP biosynthesis [34–36]. In *S. cerevisiae*, there is an additional acetyl-CoA anaplerotic route from pyruvate. PDH converts pyruvate into acetaldehyde, and the latter can be further oxidized to acetate by aldehyde dehydrogenase (ALDH). Eventually, ACS catalyzes acetate to improve cytosolic acetyl-CoA pool (Figure 2) [29,37]. In *S. cerevisiae*, the 3HP titer was increased by 80% by overexpression of the above three enzymes [38].

To block the glyoxylate cycle, Chen *et al*. deleted the *MLS1* gene in *S. cerevisiae*, encoding cytosolic malate synthase. In parallel, they overexpressed two enzymes in the acetyl-CoA anaplerotic route, including ALDH and a modified ACS. The 3HP titer was three times higher when compared with the control strain [21].

Moreover, there are various regulation factors controlling central carbon metabolism [39], such as CsrB, SgrS and ArcA [40–42]. Manipulation of those regulating systems is also an effective way to channel carbon flux into 3HP biosynthesis. For example, deletion of the *arcA* gene significantly repressed the acetate accumulation and improved the 3HP production 2-fold [43].

Redirecting carbon flux from malonyl-CoA to 3HP

Malonyl-CoA is an important precursor in the biosynthesis of fatty acids and some other compounds like flavonoids [44,45]. To redirect more malonyl-CoA into 3HP production, the antibiotic cerulenin, which can inhibit the activity of 3-oxoacyl-ACP synthase I and II [46,47], was used to suppress fatty acids biosynthesis [48].

In this study, the 3HP sensor was designed and applied to monitor real-time 3HP concentration in the culture by directing the dosage of cerulenin. Finally, under the optimal conditions, the 3HP titer was more than 20 times higher than that in previous report. However, 3HP scale production could hardly rely on the cerulenin because of a high expense [48]. Fortunately, there are some other methods such as antisense RNAs [49] and temperature sensitive mutants [50,51], which can also repress fatty acids biosynthesis conditionally and will help to increase carbon flow to 3HP biosynthesis in the future.

Improving catalysis of key enzymes

The properties and expression of the enzyme involved in a metabolic pathway have a direct impact on productivity of the engineering strains. To enhance chemical production, catalysis of key enzymes should be improved for decreased mass transfer limitations, disinhibition of precursors or products and increased activity [52].

Overcoming malonyl-CoA restriction

The generation of malonyl-CoA is catalyzed from acetyl-CoA by ACC which is a rate-limiting enzyme in fatty acid synthesis [53]. Generally, the malonyl-CoA is maintained at a low level in wild-type cells [45,54–56], which becomes a barrier in the 3HP production.

The endogenous ACC was firstly overexpressed during the fatty acid biosynthesis in recombinant *E. coli* [53]. Also, this approach was used in the malonyl-CoA pathway of 3HP with the addition of biotin and HCO_3^- during the fermentation to improve the catalysis of ACC [15,19,21,22,57]. However, cell viability could be suppressed by overexpression of the native ACC with unclear mechanism [45,53], so Cheng et al. expressed ACC from *Corynebacterium glutamicum* in *E. coli*, resulting in tremendously increased 3HP titer [58]. In *S. cerevisiae*, the activity of cytosolic ACC could be inhibited by phosphorylation, and this inhibition can be eliminated by site-directed mutagenesis of the *acc*1 gene, leading to significant enhancement of malonyl-CoA supply and 3HP titer [38,59]. Recently, a spectrophotometric assay was described for ACC activity determination, eliminating the radioactive substrate in the previous ACC assay, and can be easily used in high-throughput screening of ACC mutants [60].

Promoting catalytic efficiency of MCR

MCR derived from *C. aurantiacus* was used in most constructions of the malonyl-CoA pathway. It catalyzes a two-step reduction from malonyl-CoA to 3HP with malonate semialdehyde as an intermediate (Figure 2) [19,25], and is most active in autotrophically grown *C. aurantiacus* at 57 °C. When expressed in *E. coli* or yeast, MCR enzymatic activity was significantly impaired by the change of temperature and physiological environments, making it a rate-limiting factor of 3HP biosynthesis [8,19].

Functional domain analysis revealed that the N-terminal region of MCR (MCR-N; amino acids 1–549) and the C-terminal region of MCR (MCR-C; amino acids 550–1219) are functionally distinct. The malonyl-CoA is reduced into malonate semialdehyde with NADPH by MCR-C, and further reduced to 3HP by MCR-N.

Unexpectedly, dissection of MCR resulted in a higher substrate affinity and catalytic efficiency as well as the increased 3HP titer in *E. coli* [19]. Moreover, separating MCR into two fragments made it possible to figure out the serious enzyme activity imbalance between MCR-N and MCR-C as MCR-C showed much lower activity and expression level than MCR-N in recombinant *E. coli* strain [19]. Then, the activity imbalance was minimized by directed evolution of the rate-limiting enzyme MCR-C and fine tuning of MCR-N expression level. Combined with culture conditions optimization, these engineering approaches increased the 3HP titer from 0.15 to 40.6 g/L, representing the highest 3HP production *via* malonyl-CoA pathway so far [20].

As distinguished from the bi-functional MCR involved in the 3HP cycle of *C. aurantiacus*, two separate enzymes, malonyl-CoA reductase and malonate semialdehyde reductase, are used from the 3HP-4HB carbon fixation cycle found in certain archaea [61–64]. These two pathways shared some chemical intermediates, but the enzymes were distinctly different, suggesting they evolved independently [65]. Recently, malonyl-CoA reductase from *Sulfolobus tokodaii* and malonate semialdehyde reductase from *Metallosphaera sedula*, were overexpressed in cyanobacteria. This strain also presented higher 3HP production than the strain carrying whole-length *mcr* gene from *C. aurantiacus* [66]. In addition, a few studies attempted to develop MCR activity optimized by other means, such as expressing the genes in a hyperthermophilic host [61–64].

All the above results indicated that the activity of MCR is an essential factor for 3HP yield *via* the malonyl-CoA pathway, and it is necessary to carry out further development to achieve higher activity of MCR.

Enhancing cofactor and energy supply

Cofactors and energy are essential for many enzymatic reactions. In the malonyl-CoA pathway, ACC requires biotin and ATP for its activity [24] and MCR utilizes NADPH as an electron donor [25]. A plentiful supply of cofactors and energy has previously been proven helpful for 3HP accumulation in the engineered strains.

Enhancing biotin and ATP supply for ACC

The enzyme activity of ACC is dependent on its biotinylation [53,59], and supplementation of biotin during the fermentation can maximize the strength of ACC. Also, overexpression of a biotinylating enzyme can improve carboxylase activity of ACC in a heterologous host, because the enzyme is responsible for ligating biotin to ACC [64]. In *Pyrococcus furiosus*, the biotin protein ligase from *M. sedula* was co-expressed with ACC, which resulted in significantly improved 3HP titer [63].

Additionally, increasing energy supply is also conducive to this ATP-driven reaction. In *S. cerevisiae*, it was identified that two genes contributed to the raise in intracellular malonyl-CoA level. The *TPI1* gene was involved in the production of glyceraldehyde-3-phosphate *via* glycolysis, which resulted in ATP generation. The *PMP1* gene encoded a subunit of the proton-ATPase PMA1, which helped to form a proton gradient

and accelerate biotin transportation. Overexpression of each gene could enhance the 3HP concentration by over 100% [57].

Enhancing cofactor NADPH supply for MCR

Generation of one mole of 3HP from malonyl-CoA requires two moles of NADPH, and the enhanced production of 3HP in the cell will obviously increase the demand for reducing power. The cofactor NADPH is usually produced by the pentose phosphate pathway (PPP) or the TCA cycle employing NADP-specific isocitrate dehydrogenase (IDH) [67,68]. Meanwhile, the transformation of NADPH from NADH can be mediated by transhydrogenases and NADH kinases to enhance the level of NADPH [69,70]. In *E. coli*, the major source of NADPH lies in the PPP and the catalysis of pyridine nucleotide transhydrogenase (PNT), each of which covers 35–45%, respectively; and IDH provides most of the rest of the supply [71,72]. The malonyl-CoA pathway was ever constructed in combination with overexpression of PNT in *E. coli*, which led to a 34% higher titer of 3HP than the control [15]. Also, the NADPH supply could be raised *via* PPP by deleting the genes encoding 6-phosphofructokinase and NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the Embden–Meyerhof–Parnas pathway [73]. In another way, by overexpressing the NADP-dependent GAPDH, 3HP concentration could be increased by 30–70% in *S. cerevisiae* [21,38]. Such similar approaches have been confirmed to increase intracellular NADPH as introducing a heterogeneous NADP-dependent dehydrogenase in place of the native NAD-dependent one [74,75].

Selecting specific host strain

An ideal host strain for chemical production should have the following characteristics: Firstly, the strain should grow rapidly in simple culture media with inexpensive feedstock and accumulate products with high efficiency. Secondly, the host is supposed to be nonpathogenic, with clear genetic backgrounds, genetically stable, easy to engineer, and highly adaptable to different conditions. According to these criteria, *E. coli*, the most widely used model bacteria, was selected as the host strain for 3HP production *via* the malonyl-CoA pathway by many research groups [15,19,20,58].

However, *E. coli* has some drawbacks such as low tolerance to acidic extracellular pH. It results in the use of large amounts of base titrant to maintain the neutral medium pH as well as requirement for strong acid in the subsequent process of converting the salt into the acid form. To reduce the cost of fermentation and downstream recovery process, *S. cerevisiae* becomes an attractive alternative host for 3HP production as it tolerates relatively low pH [21].

On the other hand, the *E. coli* and *S. cerevisiae* systems need organic carbon as substrates, which may compete with world food supply. To develop more sustainable alternative carbon source for 3HP, photosynthetic cyanobacteria have attracted significant recent attention due to their ability to absorb carbon dioxide as the sole carbon source. Consequently, malonyl-CoA pathway genes were introduced into cyanobacterium *Synechocystis*, leading to 3HP production directly from sunlight and CO_2 [22,66]. Although the 3HP titer is relatively low, these studies illustrated the feasibility of photosynthetic production as a promising alternative to the traditionally biomass-based 3HP production.

Conclusion and prospects

Here, we summarize the up-to-date progress in 3HP biosynthesis *via* the malonyl-CoA pathway (Table 1). In recent years, this pathway has been successfully constructed in various species including *E. coli*, yeast and cyanobacteria, and optimized through carbon flux redirection, enzyme screening and engineering, and the increased supply of energy and cofactors, resulting in significantly enhanced 3HP titer up to 40 g/L and showing the feasibility of low-cost biosynthesis of 3HP or its derivatives and polymers at the industrial level in future.

Despite progress, the pathway performance is still unsatisfactory and there are some obstacles to be overcome in technical and commercial viability. Low yield still remains a bottleneck in the industrialization of microbial 3HP production *via* the malonyl-CoA pathway. To solve this problem, efforts should be addressed in the following areas: Firstly, ACC and MCR are essential in 3HP production, and it is necessary to continue the screening and engineering of ACC and MCR to achieve higher activity. Besides directed evolution, rational design is also a powerful vehicle for the proteins with available structure information. The structures of ACCs from various species and malonyl-CoA reductase from *S. tokodaii* have been reported recently [78–81]. With the advance in structural biology, crystal structure of more proteins involved in the malonyl-CoA pathway will be determined and help to design enzymes with desirable properties.

Secondly, acetyl-CoA and malonyl-CoA are precursors of 3HP and a large amount of other metabolites, and it is plausible that their availability is limiting for 3HP production. Several strategies have been reported to balance the precursor supply for enhanced biosynthesis of various chemicals, such as regulating malonyl-CoA

Table 1. 3-Hydroxypropionate production *via* malonyl-CoA pathway with different strategies.

Engineering strategy[a]	Host[b]	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Reactor	Carbon Source	Refs.
Increasing reducing equivalent	Ec	0.19	-	8.03E-3	Shake flask	Glucose	[15]
Dissecting MCR into two fragments with improved overall enzyme activity	Ec	0.15	-	3.13E-3	Shake flask	Glucose	[19]
Modifying cytosolic ACC activity	Sc	0.28	-	5.58E-3	1 L Bioreactor	Glucose	[59]
Increasing reducing equivalent and carbon flux toward acetyl-CoA	Sc	0.46	-	6.43E-3	Shake flask	Glucose	[21]
Expressing two independent enzymes to convert malonyl-CoA into 3HP, and heterologous biotin protein ligase and carbonic anhydrase to increasing ACC activity in a hyperthermophile strain	Pf	0.28	-	5.40E-3	3 L Bioreactor	Maltose	[64]
Increasing the intracellular concentrations of ATP and biotin to promote malonyl-CoA synthesis	Sc	>1	-	>0.01	Shake flask	Glucose	[57]
Expressing two independent enzymes from different species to convert malonyl-CoA into 3HP	Se	0.66	-	1.72E-3	Shake flask	CO2	[66]
Optimizing protein expression and increasing reducing equivalent and carbon flux	Ss	0.84	-	5.81E-3	Shake flask	CO2	[22]
Developing a dynamic regulation system between carbon flux and enzyme expression, based on a malonyl-CoA biosensor and a glucose concentration sensitive promoter	Sc	0.80	0.05	0.03	Bioreactor	Glucose	[76]
Expressing heterologous ACC and optimizing host strains and IPTG addition	Ec	1.80 10.08	0.18 -	0.04 0.40	Shake flask 5 L Bioreactor	Glucose	[58]
Tuning activity level of MCR-N and MCR-C to achieve functional balance between enzymes	Ec	3.72 40.60	- 0.19	0.08 0.56	Shake flask 5 L Bioreactor	Glucose	[20]
Developing a 3HP biosensor to optimize concentration of cerulenin and IPTG	Ec	4.20	-	0.26	96-well plate	Glucose	[48]
Expressing a modified ACC1 in the genetically stable strains and increasing reducing equivalent and carbon flux toward acetyl-CoA	Sc	3.60 9.80	- 0.07	0.05 0.10	96-well plate 1 L Bioreactor	Glucose	[38]
Expressing heterologous biotin protein ligase without carbonic anhydrase in a hyperthermophile strain	Pf	0.37	-	-	3 L Bioreactor	Maltose	[63]
Optimizing protein expression and fermentation process, and increasing reducing equivalent and carbon flux	Ec	48.4	0.53	0.23	Bioreactor	Glucose	[77]

[a]All the engineering strategies are based on overexpression of acetyl-CoA carboxylase and malonyl-CoA reductase.
[b]Ec: *Escherichia coli*; Sc: *Saccharomyces cerevisiae*; Pf: *Pyrococcus furiosus*; Se: *Synechococcus elongatus*; Ss: *Synechocystis* sp.

metabolism *via* synthetic antisense RNA [49], construction of malonyl-CoA responsive sensor [82], and a dynamically regulated pathway [76]. These strategies will greatly facilitate high-yield 3HP production in the future.

3HP toxicity also serves as a key barrier to commercialization, and the mechanism remains largely uncharacterized [83]. A titer of 50–100 g/L is expected for the economical fermentative production of most building block acids, and 3HP, at that concentration would lead to a pH reduction to around 2.0 [84]. Furthermore, undissociated 3HP can pass freely through the cellular membranes, and would dissociate in the slightly alkaline cytoplasm resulting in release of protons which lower internal pH and anions that repress some aspects of the metabolism specifically [85]. In recent years, more genes associated with 3HP tolerance were identified [85–88], and this will principally promote the development of 3HP-resistant strains.

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Disclosure statement

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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