Review

Heterologous Production of Acrylic Acid: Current Challenges and Perspectives

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Abstract: Acrylic acid (AA) is a chemical with high market value used in industry to produce diapers, paints, adhesives and coatings, among others. AA available worldwide is chemically produced mostly from petroleum derivatives. Due to its economic relevance, there is presently a need for innovative and sustainable ways to synthesize AA. In the past decade, several semi-biological methods have been developed and consist in the bio-based synthesis of 3-hydroxypropionic acid (3-HP) and its chemical conversion to AA. However, more recently, engineered Escherichia coli was demonstrated to be able to convert glucose or glycerol to AA. Several pathways have been developed that use as precursors glycerol, malonyl-CoA or β-alanine. Some of these pathways produce 3-HP as an intermediate. Nevertheless, the heterologous production of AA is still in its early stages compared, for example, to 3-HP production. So far, only up to 237 mg/L of AA have been produced from glucose using β-alanine as a precursor in fed-batch fermentation. In this review, the advances in the production of AA by engineered microbes, as well as the hurdles hindering high-level production, are discussed. In addition, synthetic biology and metabolic engineering approaches to improving the production of AA in industrial settings are presented.

Keywords: acrylic acid biosynthesis; 3-hydroxypropionic acid biosynthesis; synthetic biology; heterologous production; biosynthetic pathways

1. Introduction

Acrylic acid (AA), also known as 2-propenoic acid, propene acid, acroleic acid or vinylformic acid, is an unsaturated carboxylic acid with the chemical formula C3H4O2 (Figure 1). AA is an industrial bulk chemical with diverse applications. This organic acid can be polymerized to form homopolymers (polyacrylic acid) or can be co-polymerized with esters (e.g., butyl acrylate, ethyl acrylate, methyl acrylate) and other vinyl monomers [1]. AA and its derivatives are used to improve tackiness, hardness or elasticity, stability, durability and shelf-life in numerous polymer formulations. For example, superabsorbent polymers derived from AA (e.g., polyacrylic acid) are used in the production of diapers and sanitary pads. In addition, AA and its derivatives are used in adhesives, sealants, water treatment, plastic additives, textiles, paints, surface coatings, surfactants, and detergents, among others, due to all their mentioned beneficial properties [2]. The AA market was USD 13.35 billion in 2019 and was estimated to reach more than USD 20.2 billion by 2027 (CAGR of 5.7%) [3]. Due to the COVID-19 pandemic, the AA market in 2020 was only USD 12 billion and the projections were revised to USD 19.2 billion by 2030 (CAGR of 4.8%) [4]. Since approximately 30 % of the total market volume is related to sanitary or personal care products, such as adult diapers, the market is expected to continue growing as the geriatric population increases. Due to all its potential and market, AA is considered an interesting bulk chemical to be produced from biomass [1,5,6].
Acrylic acid and its derivatives’ chemical structures.

AA is mostly produced from the oxidation of propene obtained from crude oil. It can be produced in a one-step low-efficient process (50–60% yield) or, preferably and more recently, in a two-step more efficient process (~90%) having as an intermediary acrolein [1,7]. Since petrochemicals are not renewable, AA cost increases as the supply of petroleum decreases. In addition to the raw materials used being non-renewable, the chemical processes are energy-intensive (high temperatures are used: 260–370 °C) [8,9], use environmentally unfriendly catalysts and lead to high CO₂ emissions (3.3–7.7 kg CO₂/kg AA) [10,11]. Moreover, acrolein is toxic and explosive [11]. Therefore, it is important to develop more sustainable production processes. In the past decades, engineered microorganisms have been demonstrated to be a suitable option to produce numerous compounds with industrially relevant interest [12–17] as the production processes are more environmentally friendly and have the potential to become more cost-effective. The use of synthetic biology and metabolic engineering approaches has allowed to construct more efficient strains able to produce heterologous compounds with higher concentrations and yields [18–24]. Tools such as clustered, regularly interspaced, short palindromic repeats–associated Cas9 (CRISPR-Cas9) have revolutionized the way microorganisms are designed as they make it possible to reconstruct complex metabolic pathways in a short period [25,26].

Bearing all this in mind, research has recently focused on AA biosynthesis [2,27–34]. In the past years, AA heterologous production has significantly evolved due to all the efforts made in the past focussing on the biosynthesis of 3-hydroxypropionic acid (3-HP), a possible intermediary compound in AA biosynthesis. 3-HP bio-based production has been intensively studied, and up to 125.93 g/L concentrations have already been obtained using different pathways and hosts [35–53]. This acid can be converted to AA by catalysis (dehydrogenation) or, more recently, by fermentation. In the past decades, various industrial parties, such as OPX Biotechnologies/Dow Chemical/Cargill/Novozymes/BASF, have constructed pilot plants for bio-based production of 3-HP and its chemical conversion to AA [43,54–57]. However, although heterologous production of 3-HP followed by chemical conversion to AA is a better option than using propene, it is still not ideal as it requires a chemical catalyst, high amounts of energy and high production costs to purify 3-HP [32]. Hence, the design of a biosynthetic pathway for AA production from renewable resources is of utmost importance. Since 2015, several pathways for AA production from simple carbon sources have been developed [2,27–34]. Some of the pathways produce 3-HP as an intermediary or do a bypass having several steps in common (Figure 2). Nevertheless, as far as it is known, industry has not implemented a biosynthetic pathway to produce AA from carbon sources, since the concentrations obtained until now are low (up to 237 mg/L of AA from glucose in Escherichia coli [34]).
Additionally, the challenges that need to be addressed so that AA heterologous production can become a viable alternative to its production from 3-HP chemical conversion are explored. Although the main focus of this paper is the heterologous production of AA, 3-HP biosynthesis is also described as this compound is often produced as an intermediary in AA biosynthesis, and even when it is not produced, the pathways share several steps. Additionally, the challenges that need to be addressed so that AA heterologous production can become a viable alternative to its production from 3-HP chemical conversion are highlighted.

2. Heterologous Production of 3-HP, a Frequent Intermediary from the AA Pathway

The 3-HP building block, besides being a precursor of AA, can be used to produce several important chemicals, such as 1,3-propanediol, acrylamide and malonic acid, among others. Therefore, the US Department of Energy considered it as a “Top Value Added Chemical from Biomass” in 2004 [5,6]. Since then, 3-HP heterologous production has been thoroughly explored.

3-HP can be naturally produced as an intermediate or an end product in low levels using different pathways [58] in Lactobacillus sp. [59,60], Chloroflexus aurantiacus [61], Metallosphaera sedula [62] and Klebsiella pneumoniae [47], among others. Since the identification of these pathways in these hosts, the heterologous production of 3-HP has been reported in different hosts (E. coli, Saccharomyces cerevisiae, Corynebacterium glutamicum, Pseudomonas denitrificans and Bacillus subtilis, among others) and optimized in endogenous producers (e.g., K. pneumoniae) using mostly three different routes: glycerol (CoA-independent), β-alanine and malonyl-CoA. These routes only need two enzymes to convert these precursors to 3-HP (Figure 3). Other routes less explored include propionyl-CoA, oxaloacetate and CoA-dependent glycerol routes [63–65]. This section does not intend to give an exhaustive review of 3-HP heterologous production (for that, please check [64,66]) but instead provides some useful insights into AA biosynthesis.
In addition to these two enzymes, the enzyme to reactivate glycerol dehydratase that in the presence of coenzyme B12 transforms glycerol into 3-hydroxypropanaldehyde (3-HPA), a dihydrololate (nicotinamide adenine dinucleotide) as electron acceptor. In this route, 1,3-propanediol is produced as a by-product [72]. Additionally, 3-HP can also be produced directly from glycerol in two steps (Figure 3) [36,52,67–70].

Figure 3. 3-Hydroxypropionic acid (3-HP) biosynthetic pathway using three different intermediaries naturally produced in microorganisms from glucose: glycerol, β-alanine and malonyl-CoA. Co-B12 represents coenzyme B12, and "Co-B12 represents damaged Co-B12. NAD+: nicotinamide adenine dinucleotide; NADH: reduced form of NAD+; NADP+: nicotinamide adenine dinucleotide phosphate; NADPH: reduced form of NADPH.

2.1. Glycerol Route

The glycerol route (CoA-independent) is probably the route most studied as 3-HP can be produced directly from glycerol in two steps (Figure 3) [36,52,67–70]. Additionally, 3-HP can also be produced from glucose using glycerol as an intermediary [32,34,35,38,39,44,45,47,51,71–74]. In this case, the carbon flux is often redirected to glycerol.

From glycerol, the biosynthetic pathway starts with the B12-dependent enzyme glycerol dehydratase that in the presence of coenzyme B12 transforms glycerol into 3-hydroxypropionaldehyde (3-HPA). The next step of this pathway is catalyzed by an aldehyde dehydrogenase that converts 3-HPA to 3-HP, with NAD+ (nicotinamide adenine dinucleotide) as electron acceptor. In this route, 1,3-propanediol is produced as a by-product [72] (Figure 4). In addition to these two enzymes, the enzyme to reactivate glycerol dehydratase, known as glycerol dehydratase reactivase, a chaperone, must be expressed to repair coenzyme B12 [58].

The glycerol route has some identified advantages and disadvantages. First, this pathway is simple as it only requires two enzymatic steps to convert glycerol to 3-HP, and consequently, the concentrations and productivities are, in general, higher than the ones obtained in malonyl-CoA and β-alanine routes [53]. The direct use of glycerol as a substrate can also be seen as an advantage as this opens the door to the use of crude glycerol. Crude glycerol is a cheap and highly available by-product from the biodiesel industry that is growing at a CAGR of 5.87% (USD 46.79 billion at the end of 2021, USD 51.48 billion by 2026) [75]. Currently, close to 40 billion tons of biodiesel per year are produced [76], leading to more than 4 billion tons of crude glycerol. This glycerol is impure (70–80%) [77] and is expensive to purify for use in the food, pharmaceutical, or cosmetics industry. Consequently, it is crucial to explore crude glycerol transformation to value-added chemicals in order to sustain price and growth in the demand for glycerol. The use of crude glycerol to produce 3-HP [45] or AA may allow not only a reduction in these building blocks’ cost but also a reduction in environmental and economic problems associated with waste storing and treatment and will contribute to the viability of biodiesel, reducing its production cost and increasing the use of renewable and sustainable energy.
Figure 4. 3-Hydroxypropionic acid (3-HP) heterologous pathway in *Escherichia coli* using the glycerol route. Enzymes in pink are from *E. coli*: AckA: acetate kinase; AdhE: bifunctional aldehyde/alcohol dehydrogenase; AldH: aldehyde dehydrogenase; GapA: glyceraldehyde-3-phosphate dehydrogenase; GlpF: glycerol facilitator; GlpK: glycerol kinase; GlpR: glycerol catabolic pathway repressor; GpsA: glycerol-3-phosphate dehydrogenase; LdhA: lactate dehydrogenase; PoxB: pyruvate oxidase; Pta: phosphate acetyltransferase; YqhD: NADPH-dependent aldehyde reductase. Enzymes in blue correspond to heterologous enzymes commonly overexpressed: DhaB: B12-dependent glycerol dehydratase; GPD: glycerol-3-phosphate dehydrogenase; GPP2: glycerol-3-phosphatase; GrdAB/DhaR: glycerol dehydratase reactivase; KGSAGH/GabD4/YcdW: aldehyde dehydrogenase. Other abbreviations: Co-B12: coenzyme B12; *Co-B12: damaged Co-B12; DHAP: dihydroxyacetone-phosphate; 1,3-BPG: 1,3-bisphosphoglycerate; G3P: glyceraldehyde-3-phosphate; GAP: glyceraldehyde-3-phosphate; NAD+: nicotinamide adenine dinucleotide; NADH: reduced form of NAD+; NADP+: nicotinamide adenine dinucleotide phosphate; NADPH: reduced form of NADPH.

Regarding the glycerol route disadvantages, as the name indicates, B12-dependent glycerol dehydratase requires vitamin B12 as a cofactor. However, not all microbes are able to produce it. For example, B12 supplementation is required when *E. coli* is used as a host. Since vitamin B12 is expensive, its supplementation would be undesirable at an industrial scale, which makes *E. coli* a less suitable host to carry this pathway compared to *K. pneumoniae* and *P. denitrificans* that naturally produce this vitamin [47,78]. There are also other problems related to the first step of the heterologous pathway. Glycerol dehydratase enzyme is sensitive to glycerol accumulation and also to oxygen [79]. Therefore, 3-HP production should be preferably performed in microaerophilic or aerobic conditions. However, low oxygen concentration impacts NAD+ regeneration that is needed for the second reaction. Another identified problem is the accumulation of the intermediary produced, 3-HPA, that is toxic to the cells if accumulated in high amounts [52,53]. Therefore, 3-HPA accumulation should be as limited as possible.
3-HP heterologous production from glycerol was first reported in *E. coli* SH254 [72]. *K. pneumoniae* is known to naturally accumulate 3-HPA [80] that can be mostly converted to 1,3-propanediol and also to 3-HP but in low amounts [81]. Therefore, the DhaB operon (*dhaB1*, *dhaB2*, *dhaB3*, *dhaB4*) from *K. pneumoniae* DSM 2026 encoding glycerol dehydratase was used to perform the first step of the heterologous pathway. The second step was catalyzed by AldH (also known as PuuC) from *E. coli* K-12 MG1655 encoding an aldehyde dehydrogenase, more specifically a γ-glutamyl-γ-aminobutyraldehyde dehydrogenase. This was the first time AldH was described to have specificity towards 3-HPA. In this study, 0.58 g/L of 3-HP was obtained from 100 mM glycerol. The concentration obtained was highly dependent on the concentration of vitamin B12 supplemented. Later [67], the concentrations using the same biosynthetic pathway were increased to 4.4 g/L through the optimization of isopropyl β-D-1-thiogalactopyranoside (IPTG) and substrate concentrations, pH and liquid-to-flask ratio. Afterwards, in a fed-batch process for 72 h, the concentrations increased to 31 g/L. This study reported an instability of DhaB enzyme in the presence of glycerol and an imbalanced activity between DhaB and AldH due a low expression of AldH. Therefore, the same research group optimized the constructed strain to eliminate the imbalance between the two enzymes [68] (Table 1). To stabilize DhaB activity, glycerol dehydratase reactivase (DhaR) from *K. pneumoniae* DSM 2026 (GDRAB) was used. GDRAB is encoded by *gdrA* (formally referred as *dhaB4*) and *gdrB* genes and can reactivate glycerol-inactivated DhaB through the replacement of damaged coenzyme B12 with a new one. To balance the enzymes, DhaB was expressed in a low-copy plasmid with GDRAB, while AldH was cloned in a high-copy plasmid. This pathway [68] allowed to improve the production by 2.5-fold compared to [72] under shake-flask conditions. In addition to AldH from *E. coli*, they also tested a different aldehyde dehydrogenase-α-ketoglutaric semialdehyde dehydrogenase, from *Azospirillum brasilense* (KGSADH), that was demonstrated to have higher catalytic efficiency. This enzyme allowed improving the 3-HP production by 2.5-fold in shake flask, and in a bioreactor, up to 38.5 g/L of 3-HP was produced [68]. In a different study, Kwak et al. [73] used DhaB and DhaR from *Lactobacillus brevis* and AldH from *E. coli* and produced 14.3 g/L of 3-HP in a 2-step fed-batch process. Glucose was used as a substrate in the growth phase, and glycerol was supplemented in the production stage as it was concluded that high concentrations of glycerol inhibit cell growth and its consumption rate. This allowed reducing the accumulation of fermentation by-products (lactate and acetate). The benefits of adding glucose as a substrate were also verified by Niu et al. [74]. AldH (KGSADH) activity was enhanced in the presence of glucose. Moreover, glucose also increased ATP availability for cell growth and NAD+ for the conversion of 3-HPA to 3-HP. Later, the strain designed by Kwak et al. [73] was genetically modified by Kim et al. [82]. The genes *glpK* and *yqhD*, encoding glycerol kinase and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent aldehyde reductase, respectively, were deleted to improve the flux to 3-HP. GlpK deviates glycerol to glycerol-3-phosphate, and YqhD converts the intermediary 3-HPA to 1,3-propanediol (Figure 4). These deletions allowed increasing the concentration from 14.3 g/L to 57.3 g/L, demonstrating the need to eliminate competing pathways to increase 3-HP production.
Table 1. Examples of the heterologous production of 3-hydroxypropionic acid (3-HP) in different hosts using the glycerol route. Studies are presented chronologically for each host. Overexpressed / deleted genes and the respective organisms of origin are specified.

| Host        | Substrate (Concentration) | Pathway Genes Overexpressed in Plasmid | Other Genes Overexpressed in Plasmid | Chassis Modification | Operational Mode; Fermentation Time | 3-HP Concentration (g/L) | Ref. |
|-------------|---------------------------|----------------------------------------|--------------------------------------|----------------------|-------------------------------------|-------------------------|-----|
| E. coli BL21 | Glycerol (500 mM) or (500 mM + feed) | Kp_dhaB; Ec_aldH | - | - | Shake-flask; 48 h Fed-batch; 72 h | 4.4 | [67] |
| E. coli BL21 | Glycerol (500 mM + feed) | Kp_dhaB; Kp_gdrAB; Ec_aldH | - | - | Fed-batch; 80 h | 38.7 | [68] |
| E. coli BL21 Star | Glucose (20 g/L + feed) and glycerol (feed) | Lb_dhaB; Lb_dhaR; Ec_aldH | - | - | 2-Step fed-batch; 60 h | 14.3 | [73] |
| E. coli BL21 Star | Glucose (20 g/L + feed) and glycerol (feed) | Lb_dhaB; Lb_dhaR; Ec_aldH | - | ΔglpK; ΔqphD | 2-Step fed-batch; 48 h | 57.3 | [82] |
| E. coli W | Glycerol (25 mM + feed) | Kp_dhaB; Kp_gdrAB; Ab_kgsadh | - | - | Fed-batch; 48 h | 41.5 | [52] |
| E. coli W3110 | Glycerol (40 g/L + feed) | Kp_dhaB; Kp_gdrAB; Ec_aldH | Ec_glpF | Δpta; ΔackA; ΔqphD; ΔgipR | Fed-batch; 35 h | 42.1 | [69] |
| E. coli W3110 | Glucose (40 g/L) and glycerol (80 g/L + feed) | Kp_dhaB; Kp_gdrAB; mutant Cn_gabD4 | - | Δpta; ΔackA; ΔqphD | Fed-batch; 40 h | 71.9 | [35] |
| E. coli BL21 Star | Glucose (13 g/L + feed) and xylose (7 g/L + feed) | Lb_dhaB; Lb_dhaR; Pa_aldH | Sc_gpp2; Sc_gpd; Ec_xylR | ΔgipK; ΔqphD; ΔptsG | Fed-batch; 70 h | 29.4 | [83] |
| E. coli BW25113 | Glycerol (200 mM) | Kp_dhaB; Kp_gdrAB; Ab_kgsadh | Ec_gapA (toggle switch) | ΔgapA; ΔqphD | Shake-flask | 6.0 | [70] |
| E. coli W | Glucose and glycerol (uncertain) | Kp_dhaB; Kp_gdrAB; Ab_kgsadh (UTR opt 3) | - | Δpta; ΔackA; ΔqphD | Fed-batch; 30 h | 40.51 | [53] |
| E. coli BL21 | Glucose (20 g/L + feed) and glycerol (30 g/L + feed) | Kp_dhaB; Kp_gdrAB; Ab_kgsadh | | | Fed-batch; 50 h | 17.2 | [74] |
| E. coli W | Glucose (20 mM) and glycerol (200 mM + feed) | Kp_dhaB; Kp_gdrAB; Ab_kgsadh (UTR opt 3) | | | Fed-batch; 50 h | 56.4 | [50] |
| E. coli BL21 Star | Glucose (13 g/L + feed) and xylose (7 g/L + feed) | Lb_dhaB; Lb_dhaR; Pa_aldH | Ec_galP; Sc_gpp2; Ec_xylR; Ec_gpaA | ΔgipK; ΔqphD; ΔptsG | Fed-batch; 70 h | 37.6 | [51] |
| E. coli W3110 | Glycerol (40 g/L + feed) or Crude glycerol (40 g/L + feed) | Kp_dhaB; Kp_gdrAB; Kp_ydkW | | ΔlacI | Fed-batch; 48 h | 76.2 | 61.0 | [36] |
### Table 1. Cont.

| Host | Substrate (Concentration) | Pathway Genes Overexpressed in Plasmid | Other Genes Overexpressed in Plasmid | Chassis Modification | Operational Mode; Fermentation Time | 3-HP Concentration (g/L) | Ref. |
|------|--------------------------|----------------------------------------|--------------------------------------|----------------------|-------------------------------------|-------------------------|------|
| E. coli BL21 Star | Glucose (13 g/L + feed) and xylose (7 g/L + feed) | *Lb_dhaB, Lb_dhaR, Pa_aldH* | *Ec_galP, Sc_gpp2, Sc_gpd, Ec_xylR* | ΔglpK; ΔyqhD; ΔptsG; ΔpuuR; *PaldH* replacement | Fed-batch; ±92 h | 53.7 | [38] |
| C. glutamicum | Glucose (50 g/L) or glucose (25 g/L) and xylose (25 g/L) | *Cn_gabD4, Kp_pduCDEGH* | *Sc_gpp2, Sc_gpd* | ΔlDH; Δpta; ΔfrdA; ΔadhE | Fed-batch; 72 h | 54.8 | [49] |
| P. denitrificans | Glycerol (100 mM + feed) or crude glycerol (100 mM + feed) | *Kp_dhaB, Kp_gdrAB, Ab_kgsadh* (mutated) | - | Δ3hpdh; Δ3hibdh; P*B12 pathway replacement | Fed-batch; 48 h | up to 102 | 65.0 | [45] |
| K. pneumoniae DSM 2026 | Glycerol (25 g/L + feed) | *Kp_puuC* | - | Δpta; ΔlDH | Fed-batch; 72 h | 83.8 | [39] |
| K. pneumoniae J2B | Glycerol (100 mM + feed) | *Kp_dhaB, Kp_gdrAB, Ab_kgsadh* | - | ΔlDH; ΔfrdA; ΔadhE | Fed-batch; 48 h | 43.0 | [37] |
| K. pneumoniae DSM 2026 | Glycerol (40 g/L + feed) | *Kp_puuC* (expressed by tandem repetitive tac promoter) | - | - | Fed-batch; 98 h | 102.61 | [47] |
| L. reuteri FXZ014 + E. coli BL21 | Glycerol (20 g/L + feed) | *Cn_gabD4, Lr_pduQ* (in E. coli) | - | - | Fed-batch; 24 h | 125.93 | [48] |

1 Genes: 3hibdh: 3-hydroxyisobutyrate dehydrogenase; 3hpdh: 3-hydroxypropionate dehydrogenase; ackA: acetate kinase; adhE: alcohol dehydrogenase; aldH: aldehyde dehydrogenase; araE: arabinose transporter; dhaB: glycerol dehydratase; dhaR: glycerol dehydratase activase; frdA: succinate dehydrogenase; gdrD: glycerol dehydratase reactivase; gabD4: aldehyde dehydrogenase; galP: galactose-proton symporter; gapA: glyceraldehyde 3-phosphate dehydrogenase; gK: glucokinase; glpF: glycerol facilitator; glpK: glycerol kinase; glpR: glycerol catabolic pathway repressor; gpd: glycerol 3-phosphate dehydrogenase; gpsA: glycerol-3-phosphate dehydrogenase; ioI: transcriptional regulator of myo-inositol; ioIT1: myo-inositol permease; kgsadh: α-ketoglutaric semialdehyde dehydrogenase; lacI: DNA-binding transcriptional repressor; ldhA: lactate dehydrogenase; pduCDEGH: diol dehydrogenase; pduQ: 1,3-propanediol oxidoreductase; ppxB: pyruvate oxidase; pta: phosphate acetyltransferase; ptsG: glucose-specific phosphotransferase system enzyme IIBC component; ptsH: phosphoenolpyruvate phosphotransferase system; puuC: aldehyde dehydrogenase; puuR: puu operon repressor; xylA: xylose isomerase; xylB: xylulokinase; xylR: DNA-binding transcriptional dual regulator; ydcW: γ-aminobutyraldehyde dehydrogenase; yqhD: NADPH-dependent aldehyde reductase. 2 Organisms–Ab: Azospirillum brasilense; Cc: Cupriavidus necator; Ec: Escherichia coli; Kp: Klebsiella pneumoniae; Lb: Lactobacillus brevis; Lr: Lactobacillus reuteri; Pa: Pseudomonas aeruginosa; Sc: Saccharomyces cerevisiae. 3 UTR opt: 5’ untranslated region optimized. 4 Reported concentrations were obtained using high-performance liquid chromatography.
Since 3-HP and 3-HPA can be toxic to cells in high amounts, especially 3-HPA, research has also focused on improving E. coli (and other hosts) tolerance to these compounds or to find more resistant strains [52]. Sankaranarayanan et al. [52] verified that 3-HP production and enzyme expression and activities significantly differ among several E. coli strains. They selected the E. coli W strain for 3-HP production as it presents a high maximum growth rate and final cell density in the presence of 3-HP. Using the previous described pathway (DhaB, GDRAB and KGSADH), they were able to produce 41.5 g/L of 3-HP. A similar concentration was obtained by [69] (42.1 g/L) using DhaB, GDRAB and AldH. In this study, four genes were also deleted: ackA (acetate kinase), pta (phosphate acetyltransferase) and yqhD in order to reduce the accumulation of 3-HP production by-products (acetate, 1,3-propanediol) and glpR (glycerol catabolic pathway repressor) to increase the flux towards glycerol. AckA and pta are responsible for the conversion of acetyl-CoA to acetate (Figure 4). GlpF (glycerol facilitator) was also overexpressed to facilitate glycerol transport in the cell.

Later, Chu et al. [35] screened several aldehyde dehydrogenase enzymes in order to identify one that allows reducing 3-HPA accumulation. While most of the enzymes tested did not present increased catalytic activity, GabD4 from Cupriavidus necator demonstrated high catalytic activity towards the pathway intermediary. Moreover, the activity was improved by site-directed mutagenesis. In addition to expressing DhaB, GDRAB and mutant GabD4, they also deleted ackA, pta and yqhD genes to direct the pathway towards 3-HP production. These modifications allowed increasing the concentrations to 71.9 g/L. Lim et al. [53] also used rational untranslated region (UTR) engineering to control the accumulation of toxic 3-HP by balancing the expression of the pathway enzymes. UTR Designer (https://sbi.postech.ac.kr/utr_designer/) was used to design UTR regions with targeted expression levels of dhaB1, dhaB2 and kgsadh. The optimal balance, correspondent to high expression of KGSADH and lower expression of DhaB1, enhanced 3-HP concentration (40.51 g/L) compared to the control and allowed obtaining a yield of 0.97 g 3-HP/g glycerol. In addition to the goal of limiting 3-HPA accumulation, Sankaranarayanan et al. [50] tested cassettes containing synthetic regulation regions composed of promoters with different strengths and bicistronic ribosome-binding sites (RBs). This allowed controlling the expression of the enzymes of interest (AldH expression was 8-fold higher than that of DhaB) and reduce the intermediary accumulation. In this study, 56.4 g/L of 3-HP was produced.

To redirect the metabolic flux of the central metabolic pathway to 3-HP production, Tsuruno et al. [70] used a metabolic toggle switch [84]. This synthetic biology approach enabled the conditional repression of gapA (encoding glyceraldehyde-3-phosphate (GAP) dehydrogenase) that led to an increase in 3-HP production. In this case, gapA was deleted and a copy was overexpressed in a plasmid. The expression was controlled by the presence of IPTG. When IPTG was present, lacI (lactose operon repressor) did not repress lac promoter expression, which led to the expression of the 3-HP pathway genes and also of tetR (tetracycline repressor) that repressed gapA expression. In its turn, lack of expression of gapA during the production phase prevented 1,3-biphosphoglycerate production from GAP, decreasing the metabolic flux to pyruvate. Since fed-batch operation mode was not used in this case, the productions (≈6 g/L) were comparatively lower to previous studies (Table 1) but significantly higher than the controls, which proves that this synthetic biology approach can be beneficial to increase the 3-HP concentration.

More recently, research has been also focusing on the use of xylose [38,51,83] as a carbon source to produce 3-HP due to its availability in agricultural residues wastes. Jung et al. [83] relieved carbon catabolite repression by deletion of ptsG (glucose-specific phosphoenolpyruvate:sugar transferring system enzyme), which represses xyl operon genes, and overexpression of XylR (xyl operon transcriptional regulator). They also deleted glpK and yqhD and overexpressed glycerol-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphatase (GPP2) genes from S. cerevisiae to direct the flux to glycerol and 3-HP biosynthesis. Finally, dhaB and dhaR from L. brevis and aldH from Pseudomonas aeruginosa were used to produce 29.4 g/L of 3-HP from xylose and glucose. However, a high amount of glycerol
was accumulated, which is not ideal as high concentrations of glycerol inhibit glycerol dehydratase enzyme. To circumvent this, the gpsA gene, corresponding to glycerol-3-phosphate dehydrogenase from *E. coli*, was overexpressed instead of GPD by Heo et al. [51] in order to decrease the accumulation of glycerol. The overexpression of galP (encoding *E. coli* galactose transporter) also led to a decrease in glycerol accumulation during glucose and xylose co-fermentation, possibly due to an increase in biomass. All these modifications increased the 3-HP concentration to 37.6 g/L [51]. Later, the same research group performed more modifications to the pathway to reduce glycerol accumulation [38]. They opted for the expression of GPD instead of gpsA but also deleted *puuR* (*puu* operon repressor gene) and replaced the AldH endogenous promoter for a stronger one. Again, these modifications led to an improvement in the 3-HP concentration (53.7 g/L) from glucose and xylose.

Finally, the use of crude glycerol for 3-HP production in *E. coli* was recently reported [36]. This study allowed obtaining the highest 3-HP concentration reported in *E. coli* (76.2 g/L from glycerol and 61 g/L from crude glycerol) with only a few genetic modifications. DhaB and GrdAB were overexpressed together with several tested AldH enzymes from different organisms. γ-aminobutyraldehyde dehydrogenase from *K. pneumoniae* (*ydcW*) was selected as it was found to be the best one to catalyze the pathway’s second step. In addition, *lacI* was deleted to constitutively express the pathway genes under *tac* or *trc* promoters without the need for supplementing IPTG. Surprisingly, other genetic modifications that were performed in previous studies did not lead to an improvement in 3-HP concentrations (e.g., deletion of *yqhD*, *ackA* and *pta*).

3-HP heterologous production using the glycerol route has also been reported in *K. pneumoniae* [37,39,47], *C. glutamicum* [49], *P. denitrificans* [44,45,78] and *B. subtilis* [85]. In one of the studies focused on 3-HP production in *P. denitrificans*, KGSADH was engineered [78]. The mutated enzyme showed higher substrate specificity and led to lower accumulation of 3-HPA and, consequently, less enzyme inactivation and higher production of 3-HP than the control. Table 1 also includes some of the most promising reports on these strains. However, these studies will not be thoroughly explored, as so far there are no reports on AA production using these hosts. Nevertheless, considering the obtained concentrations (up to 102 g/L), and the fact that they naturally synthesize vitamin B$_{12}$ (and are easier to engineer to increase its availability), these hosts may be selected in the future for AA production. From these three strains, *P. denitrificans* would be the most appealing since it does not present a biosafety hazard, such as *K. pneumoniae*, and similar concentrations were reported. For this reason, the work regarding 3-HP production in this organism has been patented [44,45]. Additionally, to improve the concentrations, a co-culture engineering strategy with *Lactobacillus reuteri* and *E. coli* was recently used [48]. *L. reuteri* is able to naturally synthesize 3-HP but with high accumulation of 3-HPA. Additionally, it can naturally synthesize vitamin B$_{12}$. Therefore, *E. coli* was modified to overexpress *gabD4* and *pduQ* (1,3-propanediol oxidoreductase from *L. reuteri*) to convert 3-HPA to 3-HP (125.93 g/L) and 1,3-propanediol (88.46 g/L), respectively [48]. This 3-HP concentration (and productivity) was higher than the maximum obtained when only *E. coli* (76.2 g/L) [36] or *P. denitrificans/K. pneumoniae* (102 g/L) [44,47] was used.

### 2.2. Malonyl-CoA Route

The malonyl-CoA route to produce 3-HP has also been thoroughly studied in *E. coli* [41–43,86,87]. Compared to the glycerol route, this route has the advantage of not needing B$_{12}$ vitamin as a cofactor. However, it is well known that malonyl-CoA availability is limited in microorganisms due to tight regulation of fatty acid biosynthesis [88–90]. This low availability limits 3-HP production, whereby most of the studies focus on increasing malonyl-CoA concentration, for example, by overexpressing the enzyme acetyl-CoA carboxylase (ACC) [41–43,86,87]. In addition, biotin and NaHCO$_3$ are also supplemented to increase malonyl-CoA availability. However, biotin, as vitamin B$_{12}$, is expensive and its use is industrially not sustainable.
From malonyl-CoA, this route needs only one enzyme, malonyl-CoA reductase (MCR), and requires two NADPH molecules to produce 3-HP (Figure 5). MCR enzyme can be found in several organisms, including *C. aurantiacus*, that assimilate CO₂ through the 3-HP cycle. This enzyme contains two distinct regions, identified as MCR-N and MCR-C according to the N- and C- terminals, respectively [91]. MCR-C catalyzes the conversion of malonyl-CoA to the intermediate malonic semialdehyde, which is further converted to 3-HP by MCR-N. The first step is rate-limiting [91].

![3-HP Pathway](image)

Figure 5. 3-Hydroxypropionic acid (3-HP) heterologous pathway in *Escherichia coli* using the malonyl-CoA route. Enzymes in pink are from *E. coli*: AccADBC: acetyl-CoA carboxylase; AckA: acetate kinase; ACS: acetyl-CoA synthase; AdhE: bifunctional aldehyde/alcohol dehydrogenase; BirA: biotinilase; FabI: enoyl-acyl carrier protein; LdhA: lactate dehydrogenase; MgsA: methylglyoxal synthase; PflB: pyruvate formate-lyase; PntAB: pyridine nucleotide transhydrogenase subunits α and β; PoxB: pyruvate oxidase; Pta: phosphate acetyltransferase; YdfG: 3-hydroxy acid dehydrogenase. Enzymes in blue correspond to heterologous enzymes commonly overexpressed: ACC/DstR1: ace­tyl-CoA carboxylase; ACS: acetyl-CoA synthase; Other abbreviations: ADP: adenosine di-phosphate; ATP: adenosine triphosphate; BCCP: biotin carboxyl carrier protein; NAD⁺: nicotinamide adenine dinucleotide; NADH: reduced form of NAD⁺; NADP⁺: nicotinamide adenine dinucleotide phosphate; NADPH: reduced form of NADPH⁺.

So far, 3-HP heterologous production using the malonyl-CoA route was reported at least in *E. coli*, *S. cerevisiae*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Synechococcus elongatus* [42,86,92–95]. Rathnasingh et al. [86] were the first to report 3-HP production in *E. coli* using this route (Table 2). In addition to overexpressing MCR from *C. aurantiacus*, they overexpressed ACC (accADBC) and BirA (biotinilase) genes to increase malonyl-CoA availability and pyridine nucleotide transhydrogenase subunits α and β (PntAB from *E. coli*) gene to convert reduced nicotinamide adenine dinucleotide (NADH) to NADPH (all genes from *E. coli* K-12). Using this strategy, they were able to produce 0.193 g/L from
glucose. In a later study [87], 3-HP was produced in *E. coli* through the overexpression of
codon-optimized MCR from *C. aurantiacus* and overexpression of ACC from *C. glutamicum*.
This ACC is simpler as it contains only two subunits, AccBC and DtR1, and is reported to
be a better option to increase malonyl-CoA bioavailability in *E. coli* possibly because it is
not feedback-inhibited as the one of *E. coli*. Using fed-batch operation mode, 10.08 g/L of
3-HP was obtained from glucose.

Liu et al. [91], who studied MCR enzyme from *C. aurantiacus*, concluded that MCR
activity increases when the two enzyme fragments MCR-C and MCR-N are separated
and that MCR-N expression is significantly higher than that of MCR-N. This separation
led to an increase of 1.5 times in 3-HP production (0.15 g/L in shake-flask fermentation)
in relation to the control carrying the full-length MCR gene. ACC from *E. coli* was also
overexpressed, and MCR fragments were codon-optimized. Later, Liu et al. [41] focused on
minimizing the imbalance previously verified in MCR-N and MCR-C expression [91]. To
do that, MCR-C enzymatic activity was improved by directed evolution and the MCR-N
expression level was decreased through the genome integration of 3 copies of the MCR-N
gene [41]. Additionally, ACC from *E. coli* was overexpressed and prpR encoding an activator
protein involved in the transcriptional regulation of the propionate catabolism operon was
deleted, which blocked the production of the by-product propionate. In this study, 40.6 g/L
of 3-HP was produced in fed-batch operation mode.

To increase 3-HP concentrations and tolerance in *E. coli*, Liu et al. [42] used the synthetic
biology tool iterative CRISPR EnAbled Trackable genome Engineering (iCREATE). This
CRISPR-based tool allows the fast construction of modified genomes through multiplex
and trackable editing and was used to test hundreds of thousands of genetic modifications
in parallel. These modifications focused on the central carbon metabolism pathway to
improve malonyl-CoA availability, 3-HP production from malonyl-CoA and pathways to
increase strain tolerance to 3-HP. In addition to overexpressing MCR, they also overex-
pressed YdFG (3-hydroxy acid dehydrogenase from *E. coli*) to perform the second step of the
pathway. RBS libraries were constructed in order to improve the expression of these genes
and also AccADBC. To decrease by-product formation (lactate, acetate, formate and ethanol)
and increase the metabolic flux toward malonyl-CoA, *ldhA* (lactate dehydrogenase), *pflB*
(pyruvate formate-lyase), *addE* (alcohol dehydrogenase), *poxB* (pyruvate oxidase), *ackA*
and *pta* were deleted. Additionally, a temperature-sensitive mutation was introduced in
*fabI* (enoyl-acyl carrier protein) in order to decrease fatty acid biosynthesis at 37 °C and
increase malonyl-CoA accumulation. Finally, several mutations were tested to increase
3-HP tolerance. Previously reported genes associated with low pH and organic acid toler-
ance, and regulators and transcription factors, were targeted. Mutations in oligopeptide
ABC transporter periplasmic binding protein (OppA), aldehyde dehydrogenase A (AldA),
serine hydroxymethyltransferase (GlyA) and fused chorismate mutase/prephenate dehy-
dratase (PheA) to increase their activity demonstrated to increase 3-HP tolerance and also
production, with OppA mutation being the one that led to a higher 3-HP concentration.
Overall, these modifications allowed constructing a strain that produced 60 times more
3-HP (30 g/L) than the control.

Due to its potential, the heterologous production of 3-HP using the malonyl-CoA
pathway has also been patented by academia [96] and industry [43] (Cargill). For example,
Lynch et al. [43] implemented several modifications in addition to MCR expression, in-
cluding ACC and *pntAB* overexpression to increase malonyl-CoA and NADPH availabil-
ity, respectively; *ackA/pta/poxB, ldhA/mgsA* (methylglyoxal synthase) and *pflB* deletions to re-
duce acetate, lactate and formate accumulation, respectively; and *fabI* mutation to indirectly
increase malonyl-CoA accumulation. Concentrations of up to 49 g/L of 3-HP in *E. coli* were
reported in this patent using these modifications [43].
Table 2. Examples of the heterologous production of 3-hydroxypropionic acid (3-HP) in different hosts using the malonyl-CoA route. Studies are presented chronologically for each host. Overexpressed/deleted genes and the respective organisms of origin are specified. Reported concentrations were obtained using high-performance liquid chromatography.

| Host             | Substrate (Concentration) | Pathway Genes Overexpressed in Plasmid | Other Genes Overexpressed in Plasmid | Chassis Modifications | Operational Mode; Fermentation Time | 3-HP Concentration (g/L) | Ref. |
|------------------|---------------------------|----------------------------------------|--------------------------------------|-----------------------|-------------------------------------|-------------------------|------|
| E. coli BL21     | Glucose (100 mM)          | Ca_mcr                                 | Ca_mcr; Ec_accABC; Ec_birA; Ec_pntAB | -                     | Shake-flask (batch); 24 h           | 0.193                   | [86] |
| E. coli BL21     | Glucose (10 g/L + feed)   | Ca_mcr; Cg_accBC; Cg_dtsR1             | Ca_mcr-n (3 copies) (codon-optimized) (ΔprpR; ΔmelR; ΔmgsA); ΔfabI (at 37°C) | Fed-batch; 36 h       | 10.08                               |                        | [87] |
| E. coli BL21     | Glucose (20 g/L + feed)   | Ca_mcr-c (codon-optimized and mutated) | Ca_accABCD                           | Fed-batch; 72 h       | 40.6                                |                        | [41] |
| E. coli K12 MG1655 | Glucose (30 g/L + feed)   | Ca_mcr; Ca_mcr-c (codon-optimized and mutated) | Ca_mcr-ν (3 copies) (codon-optimized) (ΔprpR; ΔmelR; ΔmgsA); ΔfabI (at 37°C) | Fed-batch; 69 h       | 49.04                               |                        | [43] |
| E. coli BW25113  | Glucose (10 g/L + feed)   | Ca_mcr; Ec_ydfG (codon-optimized and mutated) | Ca_mcr; Ca_mcr-c (mutated); other mutations to increase 3-HP tolerance | Fed-batch; 72 h       | 30.0                                |                        | [42] |
| S. cerevisiae CEN.PK102-5B | Glucose (22 g/L + feed) | -                                      | Ca_mcr; Sc_ALD6; Sc_PDC1; Sc_ACC1 (mutated); Sc_ACS; Cla_GAPDH (mutated) (multiple copies) (all genes codon-optimized) | Fed-batch; 100 h      | 9.8                                 |                        | [92] |
| S. pombe A8      | Glucose (20 g/L) and acetate (20 mM) | -                                      | Ca_mcr (codon-optimized); cut6p       | Shake-flask (batch); 30 h | 7.6                                 |                        | [93] |
| P. pastoris X-33 | Glycerol (40 g/L + feed)  | -                                      | Ca_mcr-c; Ca_mcr-ν; Yl_ACC; Sc_cPOSS (all genes codon-optimized) | Fed-batch; 45.5 h     | 24.75                               |                        | [95] |

1 Genes: ACC1: acetyl-CoA carboxylase; ACCABCD: acetyl-CoA carboxylase; accABC: subunit of acetyl-CoA carboxylase; ackA: acetate kinase; ACS: acetyl-CoA synthase; adhE: alcohol dehydrogenase; ALD6: aldehyde dehydrogenase; birA: biotinilase; cut6p: cytosolic NADH kinase; dtsR1: subunit of acetyl-CoA carboxylase; fabI: enoyl-acyl carrier protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ldhA: lactate dehydrogenase; mcr: malonyl-CoA reductase; mcr-n: N fragment of malonyl-CoA reductase; melR: DNA-binding transcriptional dual regulator; mgsA: methylglyoxal synthase; mlrA: mannitol-specific phosphotransferase system enzyme II; PDC1: pyruvate decarboxylase; pfB: pyruvate formate-lyase; pntAB: pyridine nucleotide transhydrogenase subunits α and β; poxB: pyruvate oxidase; pta: phosphate acetyltransferase; prpR: DNA-binding transcriptional dual regulator; ydfG: 3-hydroxyacid dehydrogenase. 2 Organisms: Ca: Chloroflexus aurantiacus; Cg: Corynebacterium glutamicum; Cla: Clostridium acetobutylicum; Ec: Escherichia coli; Se: Salmonella enterica; Yarrowia lipolytica. 3 Reported concentrations.
As in *E. coli*, most studies related to 3-HP production in yeast (e.g., *S. cerevisiae*, *S. pombe*, *P. pastoris*) using the malonyl-CoA route use MCR and focus on increasing malonyl-CoA and cofactor supply [65]. In general, malonyl-CoA availability is improved by overexpressing the host ACC [92–94]. Other genes to increase acetyl-CoA supply may also be expressed, such as pyruvate decarboxylase (PDC1), acetyl-CoA synthase (ACS) and aldehyde dehydrogenase (ALD6) genes [92]. To improve NADPH availability, GAPN or GAP dehydrogenase (GAPDH) genes, homologues of *gapA*, are frequently engineered [92,94]. However, even when expressing multiple copies of these genes in the genome, the concentrations obtained in *S. cerevisiae* and *S. pombe* did not surpass 9.8 g/L [92], which is significantly lower than that reached in *E. coli*. Notwithstanding, recently, 3-HP production in *P. pastoris* reached 24.75 g/L from glycerol through the overexpression of MCR, ACC and cPOS5, a cytosolic NADH kinase used to increase NADPH availability [95]. This concentration is still lower than the one obtained in *E. coli*, but *P. pastoris* is able to efficiently use crude glycerol without purification as a substrate [97], which can also be highly advantageous.

### 2.3. β-Alanine Route

The β-alanine route is the third route explored to produce 3-HP. First, β-alanine is converted to malonic semialdehyde by β-alanine aminotransferase. In this step, pyruvate is consumed and L-alanine is produced. Then, as in the malonyl-CoA route, malonic semialdehyde is transformed to 3-HP by MCR or YdfG (Figure 6).

**Figure 6.** 3-Hydroxypropionic acid (3-HP) heterologous pathway in *Escherichia coli* using the β-alanine route. Enzymes in pink are from *E. coli*: AspA: aspartase; FumAC: fumarase A and C; FumB: fumarase B; PPC: phosphoenolpyruvate carboxylase; YdfG: 3-hydroxy acid dehydrogenase. Enzymes in blue correspond to heterologous enzymes commonly overexpressed (including enzymes expressed when *Saccharomyces cerevisiae* is the host): AAT2: aspartate aminotransferase; BAPAT/PA0132: β-alanine aminotransferase/β-alanine pyruvate transaminase; MCR: malonyl-CoA reductase; PanD: aspartate-1-decarboxylase; PYC1/PYC2: pyruvate carboxylases. Other abbreviations: NADP⁺: nicotinamide adenine dinucleotide phosphate; NADPH: reduced form of NADP⁺; TCA: tricarboxylic acid.
This route has been explored mostly in *S. cerevisiae* [40,98,99] but also in *E. coli* [46] (Table 3). However, although this route was academically less explored than the other two routes, in comparison, more patents have been filed by Cargill and Novozymes using *E. coli*, *S. cerevisiae* or *Issatchenkia orientalis* as the host (e.g., [100–103]).

*S. cerevisiae* was the first organism to heterologously produce 3-HP from β-alanine [98]. Aminotransferase from *Bacillus cereus* (BAPAT) and YdfG (from *E. coli*) were used to convert β-alanine to 3-HP. Additionally, *S. cerevisiae* aspartate aminotransferase (AAT2), pyruvate carboxylases (PYC1/PYC2) and *Triboleum castanum* aspartate-1-decarboxylase (PanD) were overexpressed with the goal of increasing β-alanine availability [40,98,99] (Figure 6). Using these genetic modifications, up to 25 g/L of 3-HP was obtained from glucose [40] and 7.37 g/L of 3-HP from xylose [99]. For *S. cerevisiae* to be able to efficiently metabolize xylose, several genes from *Pichia stipitis* were overexpressed (Table 3).

3-HP highest production via β-alanine was obtained in *E. coli* (31.1 g/L) [46]. β-alanine was converted to malonic semialdehyde by β-alanine pyruvate transaminase from *P. aeruginosa*, and malonic semialdehyde was converted to 3-HP by YdfG from *E. coli*. PanD from *C. glutamicum* and *E. coli* aspartase (AspA) and phosphoenolpyruvate carboxylase (PPC) were also overexpressed to increase the flux to β-alanine (Figure 6). The fumarases genes (*fumAC*, *fumB*), which convert fumarate to malate, were deleted to increase fumarate availability and, consequently, aspartate and β-alanine. The isocitrate lyase repressor (*iclR*) gene was deleted to improve the flux through the glyoxylate shunt. The flux towards fumarate and aspartate was also increased by replacing succinate:quinone oxidoreductase (SdhC) and AspA endogenous promoters by stronger ones.

*I. orientalis* was also demonstrated to be a suitable host to produce 3-HP using the β-alanine route as it produced 22.8 g/L in fed-batch operation mode [101]. In this patent, PanD from the insect *Aedes aegypsi* was expressed. The other genetic modifications were not specified in detail but included the expression of aspartate aminotransferases, β-alanine aminotransferases and dehydrogenases, among others, and the deletion of genes that deviated the pathway to other undesirable compounds.

The 3-HP concentrations obtained using this route (up to 31.1 g/L in *E. coli*) are, in general, lower than the ones obtained by the other two routes (up to 76.2 g/L in the glycerol route and 49.04 g/L in the malonyl-CoA route in *E. coli*). Nevertheless, this route is much less explored. In the future, the implementation of other genetic modifications or the use of other enzymes evaluated in other hosts may allow increasing the concentrations. For example, the overexpression of aspartate aminotransferase was predicted to increase in silico the concentrations in *E. coli* using this route [104], and the same was demonstrated for *S. cerevisiae* in vivo [40,98]. In addition, the deletion of *ldhA*, *poxB*, *pta*, *ackA* and/or *adhE* proved to be beneficial, in general, in the other routes since these deletions allow eliminating competing pathways that deviate pyruvate from the target route.
Table 3. Examples of the heterologous production of 3-hydroxypropionic acid (3-HP) in different hosts using the β-alanine route. Studies are presented chronologically for each host. Overexpressed/deleted genes 1 and the respective organisms 2 of origin are specified.

| Host           | Substrate (Concentration) | Pathway Genes Overexpressed in Plasmid | Other Genes Overexpressed in Plasmid | Chassis Modifications | Operational Mode; Fermentation Time | 3-HP Concentration (g/L) 3 | Ref. |
|---------------|---------------------------|----------------------------------------|--------------------------------------|-----------------------|-------------------------------------|-----------------------------|-----|
| S. cerevisiae CEN.PK102-5B | Glucose (44 g/L + feed) | -                                      | -                                    | Bc_bapat; Ec_ydfG; Sc_PYC1; Sc_PYC2; Sc_AAT2; Tc_panD (multiple copies) | Fed-batch; 80 h              | 13.7                        | [98]|
| S. cerevisiae CEN.PK 113-7D | Xylose (20 g/L + feed)   | -                                      | -                                    | Bc_bapat; Ec_ydfG; Sc_PYC1; Sc_PYC2; Tc_panD; Ps_xyl1; Ps_xyl2; Ps_xyl3 | Fed-batch; 120 h             | 7.37                        | [99]|
| S. cerevisiae CEN.PK102-5B | Glucose (10 g/L + feed) (phosphorus limitation) | -                                      | -                                    | Bc_bapat; Ec_ydfG; Sc_PYC1; Sc_PYC2; Sc_AAT2; Tc_panD (multiple copies) | Fed-batch (exponential feeding ramp); 70 h | 25   | [40]|
| E. coli W3110 | Glucose (20 g/L + feed) | Pa_pa0132; Ec_ydfG                     | Cg_panD; Ec_aspA; Ec_ppc              | ΔfumAC; ΔfumB; iclR; PsdhC and PaspA replacement | Fed-batch; 49 h               | 31.1                        | [46]|

1 Genes: AAT2: aspartate aminotransferase; aspA: aspartase; bapat: aminotransferase; fumAC: fumarase A and C; fumB: fumarase B; iclR: isocitrate lyase repressor; pa0132: β-alanine pyruvate transaminase; panD: aspartate 1-decarboxylase; ppc: phosphoenolpyruvate carboxylase; PYC1/PYC2: pyruvate carboxylases; sdhC: succinate:quinone oxidoreductase; xyl_1: xylose reductase; xyl_2: xylitol dehydrogenase; xyl_3: xylulokinase; ydfG: 3-hydroxy acid dehydrogenase. 2 Organisms: Cg: Corynebacterium glutamicum; Bc: Bacillus cereus; Ec: Escherichia coli; Pa: Pseudomonas aeruginosa; Ps: Pichia stipitis; Sc: Saccharomyces cerevisiae; Tc: Tribolium castanum. 3 Reported concentrations were obtained using high-performance liquid chromatography.
3. Heterologous Production of AA

Due to its toxicity to the cells, there are few organisms that are able to produce AA [105]. Usually, AA is present in a different form, as acrylyl-CoA (AA-CoA) that is an intermediate of 3-HP cycle in several organisms and it is then converted to propanoyl-CoA [106,107]. AA heterologous production may be or may not be via 3-HP [2,27–34]. So far, several pathways were described, two pathways via 3-HP (malonyl-CoA and glycerol routes) and three that do not synthesize 3-HP as intermediary and use β-alanine or glycerol as precursors. Since the precursors are the same, many of the reactions are the same as those previously described in Section 2, although 3-HP is not produced. Therefore, several lessons learnt from 3-HP heterologous production have been applied in AA biosynthesis. Due to all the industrial potential, several patents have been filed regarding AA heterologous production [27–30].

3.1. Using 3-HP as an Intermediary

3-HP is converted to AA using three enzymes (Figure 7a): a CoA transferase to convert 3-HP to 3-hydroxypropionyl-CoA (3-HP-CoA), a 3-HP-CoA hydratase to convert 3-HP to AA-CoA and a CoA hydrolase to convert AA-CoA to AA. The first two steps of this pathway are based on the 3-HP cycle found in several organisms, such as C. aurantiacus, Acidianus brierleyi, Acidimius ambivalens, M. sedula, Megasphaera elsdenii, Sulfolobus sp. strain VE6 and Sulfolobus metallicus [105–107]. Some of these organisms have already been explored as a source of enzymes for this pathway, but there are still several enzymes than can be evaluated.

The first study reporting AA heterologous production was by Chu et al. [32] in 2015. AA was produced in E. coli from glucose using the glycerol route, while producing 3-HP as an intermediary. Glycerol was converted to 3-HP by the expression of dhaB, gdrAB and gabD4, which were integrated into the chassis genome by homologous recombination (Table 4). Next, several genes from different organisms were screened for the three steps from 3-HP to AA. The enzymes that performed best were overexpressed in a plasmid: YciF (CoA transferase) from C. necator was used to convert 3-HP to 3-HP-CoA, Aflv_0566 (CoA dehydratase) from Anoxybacillus flavithermus to convert 3-HP-CoA to AA-CoA and YciA (CoA thioesterase) from E. coli to convert AA-CoA to AA. To increase the flux towards glycerol, the gpsA gene from E. coli and gpp2 from S. cerevisiae were overexpressed in a plasmid. Additionally, pta, ackA and yqhD were deleted to reduce the production of the by-products acetate and 1,3-propanediol and, consequently, increase the 3-HP concentration. Using this strain, it was possible to produce 0.12 g/L of AA from glucose. In addition, 2.2 g/L of 3-HP was produced, which is quite low compared to the concentrations previously described using this route (Section 2.1). This might be related to the integration of the pathway genes required to produce 3-HP from glycerol into the E. coli genome that decreases the number of copies to one. This AA production method was patented by Chu et al. [29].

Later, Rogers et al. [33] used the malonyl-CoA route via 3-HP to produce AA. They constructed an AA biosensor based on fluorescence to evaluate the several strains they constructed using high-throughput methods. The biosensor was based on acuR, a transcription regulator from Rhodobacter sphaeroides that responds to AA presence. To convert 3-HP to AA-CoA, they used a truncated version of propionyl-CoA synthase (pcs) from C. aurantiacus. The original version of this enzyme allows converting 3-HP to 3-HP-CoA, 3-HP-CoA to AA-CoA and then AA-CoA to propionyl-CoA. Therefore, the functional domain responsible for the last step was removed. To convert AA-CoA to AA, they co-expressed AA-CoA hydrolase (ach) from Acinetobacter baylyi. 3-HP was produced from malonyl-CoA by mcr. To improve malonyl-CoA accumulation, cerulenin was supplemented in 96-well plates. Cerulenin inhibits fatty acid biosynthesis, which in turn leads to an increase in malonyl-CoA availability. However, this approach is not sustainable at a higher scale as cerulenin is expensive. Nevertheless, they demonstrated that malonyl-CoA availability limits AA production as much higher concentrations were obtained when cerulenin was supplemented. Up to 4.32 mg/L of AA was produced. This reinforces that this route
can only be successfully used in a strain engineered to increase malonyl-CoA availability. Afterwards, Liu and Liu [31] also produced AA from glucose via malonyl-CoA and 3-HP mostly using enzymes from *M. sedula*: To convert malonyl-CoA to 3-HP, an MCR and an malonate semialdehyde reductase (MSR) were used; then, a 3-hydroxypropionyl-CoA synthetase (3HPCS) was used to convert 3-HP to 3-HP-CoA and a 3-hydroxypropionyl-CoA dehydratase (3HPCD) to synthesize 3-HP-CoA from AA-CoA. Succinyl-CoA synthase (SucC) from *E. coli* was used to remove the CoA moiety from AA-CoA. Again, the concentrations obtained (13.28 mg/L) using the malonyl-CoA route were low possibly due to the low accumulation of malonyl-CoA, as the strain was only improved to limit acetate formation through the deletion of *poxB*, *pta* and *ackA*.

![Possible routes for acrylic acid (AA) biosynthesis.](image)

**Figure 7.** Possible routes for acrylic acid (AA) biosynthesis. (a) Route that synthesizes 3-hydroxypropionic acid (3-HP) as an intermediary. (b-d) Routes based on (a) with fewer steps. 3-HPA: 3-hydroxypropionaldehyde; 3-HP-CoA: 3-hydroxypropionyl-CoA; AA-CoA: acrylyl-CoA; Co-B₁₂: coenzyme-B₁₂; *Co-B₁₂*: damaged Co-B₁₂; NAD⁺: nicotinamide adenine dinucleotide; NADH: reduced form of NAD⁺.
Table 4. Heterologous production of acrylic acid (AA) in *Escherichia coli* using different routes. Studies are presented chronologically. Overexpressed/deleted genes and the respective organisms of origin are specified.

| Host         | Substrate (Concentration) | Route                | Pathway Genes Overexpressed in Plasmid | Other Genes Overexpressed in Plasmid | Chassis Modifications | Operational Mode; Fermentation Time | Concentration (mg/L) | Ref. |
|--------------|---------------------------|----------------------|----------------------------------------|--------------------------------------|-----------------------|-------------------------------------|-----------------------|------|
| *E. coli* W3110 | Glucose (unknown) | Glycerol (via 3-HP) | *Cn_ydiF; Af_aflv_0566; Ec_yciA* | *Ec_gpsA; Sc_gpp2* | *Kp_dhaB; Kp_gdrAB; Cn_gabD4; Δpta; ΔackA; ΔyqhD* | Shake-flask (batch); 15 h | 120 | [32] |
| *E. coli* DH5x | Glucose (50 mM) | Malonyl-CoA (via 3-HP) | *Ca_mcr; Ca_pcs* (truncated); *Ab_ach* | - | - | 96-well plate (batch); 12 h (w/ cerulenin supply) | 4.32 | [33] |
| *E. coli* K12 MG1655 | Glucose (20 g/L + feed) | Malonyl-CoA (via 3-HP) | *Ms_mcr* (codon-optimized); *Ms_msr; Ms_3hpcs; Ms_3hpcd; Ec_sucCD* | - | ΔpoxB; Δpta; ΔackA | Fed-batch; 24 h | 13.28 | [31] |
| *E. coli* BL21 | Glycerol (20 g/L) | Glycerol (not via 3-HP) | *Ca_pcs* (truncated, mutated); *St_pduP* | - | *Kp_gdrAB; Kp_dhaB; ΔprpR* | Shake-flask (batch); 48 h | 37.7 | [2] |
| *E. coli* K12 MG1655 and glucose (1 g/L) | Glycerol (30 g/L) | Glycerol (not via 3-HP) | *Ip_dhaB; Ip_gdrAB; Le_pduP; Me_MELS_1449; Ec_yciA* | - | Δpta; ΔackA; ΔyqhD | Fermenter (batch); 48 h | 44 | [27] |
| *E. coli* W3110 | β-alanine (not via 3-HP) | Glycerol (10 g/L + feed) | *Cp_act; Cp_ac12; Ec_yciA* | *Cg_panD; Ec_aspA* | ΔyhdH; ΔfumAC; ΔfumB; ΔicdR; P_ova and P_as replacement | Shake-flask (batch); n/d | 60 | 237 [34] |
| *E. coli* BL21 | Glycerol (10 g/L) | Glycerol (not via 3-HP) | *Kp_dhaB; Go_3had* | - | - | Shake-flask (batch); 32 h | 144 | 105 [34] |

1 Genes: 3had: 3-hydroxyacyl-(ACP) dehydratase; 3hpcd: 3-hydroxypropionyl-CoA dehydratase; 3hpcs: 3-hydroxypropionyl-CoA synthetase; *ach*: acrylyl-CoA hydrolase; *ackA*: acetate kinase; *acl2*: β-alanyl-CoA:ammonia lyase; *acs*: acetyl-CoA synthetase; *act*: β-alanine CoA transferase; *af习俗*: CoA dehydratase; *aspA*: aspartase; *dhaB*: glycerol dehydratase; *fumAC/B*: fumarase AC/B; *gabD4*: aldehyde dehydrogenase; *gdrAB*: glycerol dehydratase reactivase; *iclR*: isocitrate lyase repressor; *mcr*: malonyl-CoA reductase; *MELS_1449*: 3-hydroxypropionyl-CoA dehydratase; *msr*: malonate semialdehyde reductase; *panD*: aspartate 1-decarboxylase; *pcs*: propionyl-CoA synthase / enoyl-CoA hydratase; *poxB*: pyruvate oxidase; *pta*: phosphate acetyltransferase; *sucCD*: succinyl-CoA synthetase; *yciA*: CoA thiosterase; *ydiF*: CoA transferase; *yhdH*: acrylyl-CoA reductase; *yqhD*: NADPH-dependent aldehyde reductase. 2 Organisms: *Ab*: Acinetobacter baileyi; *Af*: Anoxybacillus flavithermus; *Ca*: Chloroflexus aurantiacus; *Cg*: Corynabaeterium glutamicum; *Cn*: Cupriavidus necator; *Cp*: Clostridium propionicum; *Ec*: Escherichia coli; *Go*: Gluconobacter oxydans; *Ip*: Ilyobacter polytropus; *Kp*: Klebsiella pneumoniae; *Lr*: Lactobacillus reuteri; *Sc*: Saccharomyces cerevisiae; *St*: Salmonella typhimurium; *Me*: Megasphaera elsdenii; *Ms*: Metallosphaera sedula. 3 Reported concentrations were obtained using high-performance liquid chromatography.
3.2. Without 3-HP as an Intermediary

Tong et al. [2] developed a pathway with several steps from the glycerol route to produce 3-HP but without producing 3-HP as an intermediary. This pathway required 4 enzymatic steps to convert glycerol to AA (Figure 7b). Glycerol was converted to 3-HPA by glycerol dehydratase, as previously described in Sections 2.1 and 3.1. Next, instead of being transformed to 3-HP, 3-HPA was directly converted to 3-HP-CoA by the enzyme propionaldehyde dehydrogenase. Afterwards, the next two steps were performed, as described in Figure 7a. Enoyl-CoA hydratase/3-HP-CoA hydratase converts 3-HP-CoA to AA-CoA, and a CoA transferase converts AA-CoA to AA. In this study, the authors used the previously described enzymes DhaB and GdrAB for the glycerol dehydratase and reactivation step. For the second step, they used a propionaldehyde dehydrogenase from Salmonella typhimurium (PduP) [108]. The last step was performed by endogenous CoA transferases, and no gene was overexpressed. The focus of the study was the enoyl-CoA hydratase step. Several enzymes were tested: PcsII, the enoyl-CoA hydratase domain of the trifunctional enzyme pcs from C. aurantiacus; PhaJ1 enoyl-CoA hydratase from P. aeruginosa; and hypothetical enoyl-CoA hydratase EcH from P. aeruginosa and HcaD from B. subtilis. The pathway with PcsII was the one that allowed obtaining higher amounts of AA. Afterwards, the catalytic activity of this enzyme was improved by comparing its sequence with other enoyl-CoA hydratases and performing direct mutagenesis. A double mutation allowed increasing the concentrations up to 37.7 mg/L. The prpR gene was also deleted to block propionate production, as in [41]. The authors also deleted yhdH that encoded an NADPH-dependent AA-CoA reductase previously described to convert AA-CoA to propionyl-CoA [109]. However, instead of increasing, the concentrations decreased significantly, as well as the strain growth. This demonstrated that the E. coli strain with this knockout is more sensitive to the presence of AA. Therefore, it was more beneficial to use a strain without the knockout that was also able to consume some of the intermediaries needed for AA production [2].

In addition, in 2016, Nam et al. [27] patented the same pathway described by Tong et al. [2]. They obtained similar concentrations (44 mg/L) but with a different combination of genes. After testing several genes for each step, they concluded that the pathway that performed better contained DhaB (DhaB1, DhaB2, and DhaB3) and GdrAB from Ilyobacter polytropus, PduP from L. reuteri, MELS_1449 (3-HP-CoA dehydratase) from Megasphaera elsdenii and YciA from E. coli [27]. Acka, Pta and YqhD genes were deleted, as in other studies [31,32].

More recently, a pathway to produce AA was developed based on the β-alanine route but also without producing 3-HP [34]. In this biosynthetic pathway, β-alanine is converted to β-alanyl-CoA by a β-alanine CoA transferase (act), which is then converted to AA-CoA by β-alanyl-CoA:ammonia lyase (acl2), both enzymes from Clostridium propionicum. Finally, several enzymes were tested to convert AA-CoA to AA, but YciA was selected, as in the other pathways, due to its superior performance. This pathway is simpler as it only requires three heterologous steps, while the pathways that produce 3-HP as an intermediary require five steps and the pathway that converts glycerol to AA without producing 3-HP requires four steps. However, to obtain up to 237 mg/L of AA, the highest concentration reported so far, the authors overexpressed several genes and performed various knockouts. One of the strain modifications was the deletion of yhdH (=acuI) to prevent AA-CoA conversion to propionyl-CoA. As reported in [2], a decrease in the bacterial growth using the ΔyhdH strain in the presence of AA was verified in comparison to the wild type. However, in this study, the deleted strain was selected to perform the production experiments as the inhibition effects reported were much lower than in [2], since after a significant lag phase, the deleted strain reached lower growth than the wild type but still significant. The iclR and fumAC/fumB genes were also deleted to increase the flux through a glyoxylate shunt and to increase fumarate availability, which, consequently, increase aspartate production, the precursor of β-alanine (Figure 7c). Then, as previously reported in pathways from Section 2.3, the AspA gene from E. coli and PanD from C. glutamicum were overexpressed to convert fumarate to β-alanine. AspA and ACS chromosomal promoters were also replaced.
by stronger constitutive promoters. ACS overexpression led to an increase in acetyl-CoA and, consequently, to an increased flux in the TCA cycle and then in β-alanine precursors. In addition, acetyl-CoA is also needed to convert β-alanine to β-alanyl-CoA. The production process using this pathway was also patented [30].

Recently, a new pathway from glycerol that produces acrolein as an intermediary was described (Figure 7d) [105]. This pathway is based on the pathway from *Glucobacter oxydans* that is able to produce 3-HP and AA using acrolein as an intermediate. In this study, the enzymes involved in acrolein production from 3-HPA were identified: two isoenzymes named 3-hydroxyacyl-(ACP) dehydratase (3HAD) and 3-hydroxydecanoyl-(ACP) dehydratase (3HDD). Next, the authors transferred parts of the identified pathway to *E. coli* to produce AA using resting cells. For the first step, glycerol dehydratase from *K. pneumoniae* was used (*dhaB*) to convert glycerol to 3-HPA. The expression of glycerol dehydratase reactivase was not mentioned. Then, 3-HPA was converted to acrolein by 3HAD and/or 3HDD from *G. oxydans*. No enzyme was expressed to convert acrolein to AA, so it is assumed that an endogenous aldehyde dehydrogenase (e.g., ALDH) performed this step. The expression of *3had* alone with *dhaB* allowed obtaining a higher AA concentration (144 mg/L in shake-flask culture) than when *3had* and *3hdd* were co-expressed. Resting cells were used in this study in order to decrease the inhibition of *E. coli* growth by AA. This is in accordance with the study by Arya et al. [110] who concluded that the AA effect is specific on growth and not in the central carbon metabolism and that, consequently, a high concentration of AA can be tolerated under resting conditions. In addition, in [105], acrolein accumulation was not observed, which is positive due to its reported toxicity. This 3-step pathway allowed obtaining the highest concentration so far in batch fermentation. Since apparently, there was no expression of a glycerol dehydratase reactivase and also no expression of a specific enzyme to convert acrolein to AA, these results are promising. It is expected that more investment in this simple pathway can lead to much higher concentrations in the future.

4. Key Points to Optimize AA Heterologous Production

Although studies presented in Section 3 represent a step forward in AA production, the concentrations are still low and more optimizations are needed at genetic and operational levels. The work developed regarding 3-HP biosynthesis allowed the rapid design of strains able to produce AA. However, several optimizations performed for 3-HP biosynthesis that allowed successful increase in the concentrations still need to be implemented in AA biosynthesis strain design. For example, to increase AA production from malonyl-CoA, it is mandatory to increase its availability, as proven in Section 2.2. Although this was extensively explored in 3-HP production, it was barely implemented in the experiments regarding AA biosynthesis [31,33]. Additionally, AA production using *E. coli* and the glycerol route with 3-HP as an intermediary would probably easily increase by directly supplementing glycerol and by overexpressing the genes from the 3-HP pathway using plasmids instead of integrating these genes in the chassis genome. Moreover, regarding AA production from β-alanine, the overexpression of more genes related to β-alanine accumulation would also help to increase the concentration (e.g., aspartate aminotransferase [40,104], phosphoenolpyruvate carboxylase [46]). Lastly, the elimination of competing pathways (e.g., deletion of *yqhD*) in the recent study by Zhao et al. [105] would also probably allow improving AA production.

In addition to the referred possible ways to improve the concentrations, other strategies are needed, for example, to solve the problem of *B₁₂* supplementation necessity in the glycerol route, to find or create more efficient enzymes and to increase the host tolerance to AA, among others. These strategies are highlighted in this section.

4.1. Testing a *B₁₂*-Independent Enzyme in the Glycerol Route

*B₁₂*-dependent glycerol dehydratase, which performs the first step in the glycerol route with or without 3-HP as an intermediary, represents a limiting step of the AA
Although the concentrations obtained with this enzyme are high, up to 76.2 g/L in *E. coli*, the use of this pathway at an industrial scale is unsustainable due to the need of supplementing B12. In addition, the engineering of the B12 pathway in *E. coli* is not ideal due to the challenge of expressing more than 20 genes [111,112]. Therefore, new enzymes ought to be discovered and tested as the glycerol pathway is the one with the most potential to produce AA (and 3-HP), and on top of that, it can use as a substrate an industry by-product (crude glycerol from the biodiesel industry).

In the past years, a B12-independent glycerol dehydratase was described for the heterologous production of 1,3-propanediol [113–115]. 1,3-Propanediol is an important building block and can be produced from 3-HPA, as previously mentioned. The step described for 1,3-propanediol production is exactly the same from the 3-HP pathway where glycerol is converted to 3-HPA. This type of B12-independent enzyme was first described in *Clostridium butyricum* [116,117] that is used for 1,3-propanediol biosynthesis. Since then, several bioinformatic studies have identified other putative enzymes, all from organisms that also contain B12-dependent enzymes [118]. B12-independent enzymes from *C. butyricum*, as B12-dependent enzymes, also contain several different units in an operon: *dhaB1*, *dhaB2* and *dhaT*. The first two encode B12-independent glycerol dehydratase and its activator factor (an S-adenosyl-L-methionine (SAM)-dependent enzyme), and *dhaT* encodes an enzyme specific for 1,3-propanediol biosynthesis [114].

It is unclear why this enzyme has not yet been used for 3-HP/AA biosynthesis. However, a patent was filed regarding glycerol conversion to 3-HP or 1,3-propanediol, where the possibility of using a B12-independent enzyme is mentioned [119]. Since this enzyme, as B12-dependent enzyme, is sensitive to oxygen (and glycerol) [117], the dissolved oxygen concentration should be in the range of 30% or less, preferably 10% or less, at the time of initiation of its expression [119]. The adjustment of the oxygen concentration can be performed by controlling the aeration and agitation rates in a bioreactor. In addition, sequential inoculation of strains carrying separate modules of the pathway may be a possibility if the oxygen demands for the different steps of the pathway are not compatible. Moreover, the expression of a B12-independent enzyme could be triggered by the absence of oxygen using the inducible *nar* promoter [119,120].

Nevertheless, the results obtained with B12-independent enzymes for 1,3-propanediol biosynthesis are encouraging (up to 104.4 g/L was produced [115]), whereby these enzymes should be used in the future in the development of an economical B12-free bioprocess for AA and even 3-HP biosynthesis using renewable substrates. In addition, the engineering of this enzyme using synthetic biology techniques, as described in the next section (Section 4.2), would be of utmost importance to decrease its sensitivity to oxygen and glycerol and in turn simplify the bioprocess using this pathway.

4.2. Unravelling More Efficient Enzymes and Pathways

Currently, there is a need to search for even more efficient enzymes. Two different approaches are important and should be used: the search for new enzymes in other organisms never considered and the modification of known enzymes to reach a desirable activity or specificity or even to eliminate some types of present regulation. As established, more is not always better, and when toxic compounds are present, such as 3-HPA, it is important to design pathways with limited accumulation of intermediaries [88]. In these cases, enzymes with different activities or the use of different promoters with different strengths is ideal to balance the pathways, as previously demonstrated [34,121–123]. New synthetic biology tools, such as CRISPRi/a (CRISPR interference/activation), can also be extremely important to balance the pathway and increase or decrease the expression of a specific enzyme [25]. The use of methods such as EvolvR, a CRISPR-Cas9-based targeted mutagenesis method [124], to create gene libraries can also be useful to modify enzymes activities. This modification is created by engineered error-prone DNA polymerases that target defined locations via CRISPR-guided nickases (nCas9) and generate mutations. Similar methods (e.g., iCREATE) were previously used to modify the *E. coli* genome and
increase 3-HP concentrations [42]. Finally, the use of scaffold proteins can also be beneficial for these cases as they help to decrease intermediary accumulation due to co-localization and also decrease the conversion of intermediaries to other undesirable by-products, such as propionyl-CoA or 1,3-propanediol in the case of the AA pathway [26,84]. Therefore, this type of strategy could be used to improve AA concentrations.

In addition to the pathways previously described in Sections 2 and 3, there are other pathways less explored to produce 3-HP and AA that can be a source of more efficient enzymes even if the pathways are not the most efficient. For example, AA-CoA, in addition to being an intermediate in the 3-HP cycle in some organisms, is also an intermediate in the fermentation of lactate by *C. propionicum*, *Propionibacterium freudenreichii*, *Clostridium homopropionicum*, *Clostridium neopropionicum* or *Veillonella parvula*, among others [125,126]. In this pathway, lactate is converted to lactoyl-CoA, which is then converted to AA-CoA. Afterwards, AA-CoA can be converted to propionyl-CoA or AA [127]. This pathway has not been explored for AA production, as the production is low and transient in the endogenous organism and only occurs in certain circumstances. Still, the enzyme that performs the last step could be evaluated as the pathway can be highly regulated in the endogenous organism. Another pathway that has been less explored for 3-HP production is the one that uses propionate as a precursor, producing as intermediates propionyl-CoA, AA-CoA, 3-HP-CoA and, finally, 3-HP [63,128]. Part of this pathway is the reverse of the pathway used to produce AA from 3-HP (Figure 7). Nevertheless, this pathway could be adapted to produce AA from propionate, having as intermediates propionyl-CoA and AA-CoA. Therefore, the enzymes previously used could be evaluated in new pathways.

4.3. Improving Chassis Tolerance to AA

It is well described in the literature that 3-HPA, 3-HP and AA are toxic to cells in high concentrations, with AA being more toxic than 3-HP [32,105]. It is known that AA presence in high concentrations increases the lag phase and decreases the bacterial growth rate and final cell concentration [34,129], demonstrating potential antimicrobial properties. Several factors contribute to AA toxicity. One factor is general to short-chain acids. These acids are known to decrease the rate of DNA, RNA, protein and lipid synthesis. They affect genes and proteins involved in the transcription-translation machinery, regulation and response to stress [130]. In addition, the AA double-bond structure can conjugate with glutathione, causing its depletion [1]. This may lead to toxicity since glutathione protects bacteria from low pH, chlorine, and also osmotic and oxidative stresses [131]. The importance of glutathione availability was demonstrated for 3-HP production in *S. cerevisiae* [132]. In addition, the AA α,β-conjugated unsaturated carbonyl group was demonstrated to be toxic to bacterial metabolism [133].

Due to the reported AA toxicity, high productions can be challenging in *E. coli* or other organisms. To improve product concentrations, several metabolic engineering strategies can be used. As previously mentioned, 3-HP tolerance was increased through the modification of the *E. coli* genome using iCREATE [42]. Genes associated with low pH, transporters, organic acid tolerance, transcription factors and regulators were targeted in [42] and other studies (e.g., [43,134]). Strategies to increase the unsaturated fatty acid content in the membrane have also been successful in increasing *E. coli* tolerance to 3-HP [135]. In addition, it was demonstrated that *E. coli* is more sensitive to AA under anaerobic conditions [110] and this sensitivity is related to the inhibition of pyruvate formate lyase (PFLB) that is required for *E. coli* anaerobic growth. Therefore, this enzyme gene could be an interesting target to increase AA tolerance. In the future, other target genes need to be identified and transcriptome and proteome studies can be useful to identify the genes affected by AA presence. After identification of the target genes, several synthetic biology tools may be used to create mutations in the selected genes, including the abovementioned EvolvR [124]. Random mutagenesis can also be used to unravel new targets. After mutagenesis, which can be performed using different methods (e.g., UV irradiation, ethidium bromide or N-methyl-N’-nitro-N-nitrosoguanidine exposure), clones can be screened for changes in the
tolerance to AA. Strains with altered tolerance profiles can then be sequenced to identify the location of the mutation using next-generation sequencing.

Additionally, AA biosynthesis can be optimized at the operational level. Different operation modes may decrease possible metabolite inhibitions and, therefore, increase the productivity. For example, in situ product removal strategies may be evaluated to remove AA from the culture broth using solid-liquid extraction methods and, consequently, avoid the AA toxic effect [156,137]. Adsorption with solid resins (e.g., Amberlite) was recently successfully used to remove AA from an aqueous medium [138]. These resins can be used directly in a fermenter since they are non-toxic to microorganisms. This approach may allow simultaneous AA production and purification. In addition, liquid-liquid extraction methods using natural oils or ionic liquids is also reported to remove AA [139,140]. Moreover, if the glycerol route is selected, the dissolved oxygen concentration should be carefully optimized due to the enzyme sensitivity to oxygen (glycerol dehydratase) and to the NAD⁺ regeneration need. Oxido-reduction potential (ORP) control could be used as an alternative approach to control the NADH/NAD⁺ ratio and avoid the contradiction between anaerobic glycerol dehydratase activity and aerobic regeneration of NAD⁺ [66,141]. The control and variation of O₂ concentration is also an important factor as E. coli has proved to be more sensitive to AA under anaerobic conditions [110], as mentioned before. Regarding pH control, it is also of upmost importance. For example, it was reported that AA inhibition in Clostridium and possibly in other bacteria is lower at a higher pH (≥ 7) [129]. The production in a bioreactor has the potential to help solve these problems as it allows obtaining a tighter control of pH, dissolved oxygen concentration and possibly ORP.

5. Concluding Remarks and Future Perspectives

AA has numerous important applications for our day-to-day life. The market is expected to grow as the population increases and ages. Therefore, more sustainable and efficient production processes ought to be developed. In the past years, there have been remarkable advances in developing artificial biosynthetic pathways for AA heterologous production in E. coli. Nevertheless, the heterologous production of AA is still in an initial stage and has a long way to go. In this paper, the reported pathways for AA biosynthesis in E. coli were thoroughly reviewed, together with 3-HP ones, as the lessons learnt during the construction of this pathway were essential to develop new ones for AA biosynthesis. The most promising pathways for AA production so far are probably the ones that use glycerol as a substrate. There are currently several variations of the glycerol route, and all have high potential, with concentrations so far reaching up to 144 mg/L in shake-flask culture. However, routes that bypass the 3-HP production step appear more promising as fewer steps are needed to reach the goal. Although the β-alanine pathway reported concentrations of up to 237 mg/L, these are obtained in bioreactors using fed-batch operation mode and the concentrations obtained in batch mode in the same study are significantly less competitive (up to 60 mg/L). Therefore, in the future, the use of a larger-scale and fed-batch process is expected to highly increase the concentrations obtained using the glycerol route. Of course, it is important to not forget the limitations of these pathways first step performed so far by a B₁₂-dependent glycerol dehydratase that represents one of the most important bottlenecks of AA (and also 3-HP) biosynthesis. As discussed in Section 4.1, it is mandatory to optimize these pathways using a B₁₂-independent glycerol dehydratase previously reported as efficient for the production of 1,3-propanediol. This is absolutely required to move to an industrial setting using this pathway, as it most probably makes no sense to select a pathway that can use the by-product crude glycerol but needs the supplementation of the expensive vitamin B₁₂. Several enzymes should be evaluated to find the best candidate to perform this step. In addition, the glycerol route that produces acrolein as an intermediary also needs to be more explored as it was the one that allowed obtaining higher concentrations and still has a large room for improvement. It is important to find the most adequate enzymes for this pathway as only two enzymes were evaluated to convert 3-HPA to acrolein and the step that converts acrolein to AA was left to an endogenous enzyme that was not overexpressed.
In the future, as some of the identified bottlenecks related to enzyme selection resolve, others will emerge and metabolic engineering and synthetic biology strategies will continue to be essential to balance the pathway (e.g., CRISPRi/a) and optimize AA production levels. Synthetic biology tools will continue to evolve and to respond to the biotechnological field challenges and contribute to the development of a sustainable, efficient and fast process for AA biosynthesis.

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