

Recent Advances and Challenges in Engineering Metabolic Pathways and Cofactor Regeneration for Enhanced n-Butanol Biosynthesis

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ABSTRACT: The biological production of n-butanol has seen renewed interest due to the need for the production of sustainable aviation fuel, for which n-butanol serves as a direct precursor. However, biological production of this alcohol is still limited by the fermentation's low titers and low yields. Many approaches have been taken to increase n-butanol production, such as using alternative host organisms, utilizing heterologous enzymes for acid reduction and cofactor regeneration, and protein engineering of critical enzymes in the n-butanol production metabolic pathway. This review highlights key achievements made in each of these areas and shows the potential for these approaches in increasing n-butanol production. The review closes by pinpointing the challenges and limitations in these approaches and recommends that the ultimate approach to n-butanol production should inevitably utilize noncanonical redox cofactors to drive metabolic flux for butanol biosynthesis from glucose.

Keywords: Aldehyde/alcohol dehydrogenase; Butanol; Carboxylic acid reductase; Clostridium; Cofactor regeneration; Metabolic engineering; Protein engineering



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1. Introduction

Among biofuels, biobutanol stands out due to its superior fuel characteristics: it contains nearly 30% more energy than ethanol and offers combustion performance comparable to gasoline. Unlike bioethanol and biodiesel, biobutanol can be directly blended with gasoline or diesel, requiring minimal or no engine modifications [1,2]. A well-known method for producing n-butanol biotechnologically is the Acetone-Butanol-Ethanol (ABE) fermentation process [3,4]. Despite its long-standing recognition, the ABE fermentation process still faces several challenges that hinder its industrial feasibility [5]. It suffers from expensive feedstock, low butanol yield, low productivity, and high operational and equipment costs, with added significant water consumption. Many efforts have been made to make the biobutanol production process more feasible [6]. These range from process engineering to microbe strain engineering [7–9]. This review provides an overview of recent advances and challenges in metabolic and protein engineering approaches made to improve the biobutanol production process focusing on the metabolically engineered *Clostridium acetobutylicum* [10], *Clostridium tyrobutyricum* [11], and *Escherichia coli* [12].

2. Metabolic Engineering for Biobutanol Production

n-Butanol is naturally produced in some solventogenic clostridia [5]. There have also been extensive efforts to engineer various microorganisms for biobutanol production [8–10]. We highlight recent works on engineering *C. acetobutylicum*, *C. tyrobutyricum*, and *E. coli* for n-butanol production in Table 1, with detailed discussion given in this section.

Table 1. Notable examples of n-butanol production by engineered strains of *C. acetobutylicum*, *C. tyrobutyricum*, and *E. coli*.

Strain	Engineering Strategy	Fermentation Conditions	Titer (g/L)	Yield (g/g)	Productivity (g/L·h)	Reference
<i>C. acetobutylicum</i> JB200	ATCC 55025 mutant obtained from adaptive evolution	Batch fermentation of glucose	20.3	0.23	0.33	[13]
<i>C. acetobutylicum</i> HKKO	ATCC 55025 with <i>cac3319</i> KO to increase butanol tolerance	Batch fermentation of glucose	18.2	0.20	0.38	[14]
<i>C. acetobutylicum</i> HKKO- Δ <i>adc-adhE2</i>	KO: <i>adc</i> ; OE: <i>adhE2</i>	Batch fermentation of glucose	19.7	0.26	0.41	[15]
<i>C. acetobutylicum</i> Δ <i>hydA</i>	ATCC 55025 with <i>hydA</i> KO to increase NADH	Batch fermentation of glucose with methyl viologen	13.8	0.28	0.31	[16]
<i>C. acetobutylicum</i> CAB1060	OE: <i>atoB</i> , <i>hbd</i> (Ck), <i>crt</i> , <i>bcd</i> , <i>etfA</i> , <i>etfB</i> , <i>adhE2</i> KO: <i>ptb</i> , <i>buk</i> , <i>ctfAB</i> , <i>ldhA</i> , <i>rexA</i> , <i>thlA</i> , <i>hbd</i>	Fed-batch fermentation of glucose with <i>in-situ</i> butanol separation by vacuum distillation	550	0.35	14	[17]
<i>C. tyrobutyricum</i> Ack- <i>adhE2</i>	OE: <i>adhE2</i> in Ack strain	Batch fermentation of glucose	10	0.27	0.03	[18]
		Mannitol	16	0.31	0.06	
			20.5	0.33	0.32	
		Glucose	14.5	0.28	0.13	[19]
		Cassava bagasse	13	0.34	0.26	[20]
		Corn fiber, cotton stalk, soybean hull, sugarcane bagasse	15	0.3	0.3	[21]
Ack- <i>adhE2-ctfAB</i>	OE: <i>adhE2</i> , <i>ctfAB</i>	Glucose	12	0.26	0.35	[22]
Ack- <i>adhE2-scrABK</i>	OE: <i>adhE2</i> , <i>scrA</i> , <i>scrB</i> , <i>scrK</i>	Sucrose	14.8	0.21	0.15	[23]
Ack- <i>adhE2-aglul</i>	OE: <i>adhE2</i> , <i>aglul</i>	Sugarcane juice	12.8	0.21	0.53	
		Maltose	17.2	0.2	0.29	[24]
Ack- <i>adhE2-xyITBA</i>	OE: <i>adhE2</i> , <i>xyIT</i> , <i>xyIA</i> , <i>xyIB</i>	Soluble starch	16.2	0.17	0.19	
		Glucose/Xylose	12	0.12	0.17	[25]
Δ <i>cat1::adhE2</i>	KO: <i>cat1</i> ; OE: <i>adhE2</i>	Soybean hull	15.7	0.24	0.29	
		Glucose	26.2	0.23	0.16	[26]
Ct-pMA12G	OE: <i>adhE2</i> , GroESL	Paper mill sludge	16.5	0.26	0.17	[27]
		Brown algae biomass hydrolysate	12.2	0.34	0.15	[28]
<i>E. coli</i> JCL187	OE: <i>atoB</i> , <i>hbd</i> , <i>crt</i> , <i>bcd</i> , <i>etfA</i> , <i>etfB</i> , <i>adhE2</i> ; KO: <i>adhE</i> , <i>ldhA</i> , <i>frdBC</i> , <i>pta</i> , <i>fnr</i>	Semi-aerobic batch fermentation with rich media supplemented with glycerol.	0.55	N/A	N/A	[29]
<i>E. coli</i> JCL299	OE: <i>atoB</i> , <i>hbd</i> , <i>crt</i> , <i>ter</i> , <i>adhE2</i> , <i>fdh</i> KO: <i>ldhA</i> <i>adhE</i> <i>frdBC</i> <i>pta</i>	Fed-batch fermentation of glucose with continuous butanol removal	15	0.28	0.18	[30]
<i>E. coli</i> EB243	OE: <i>atoB</i> , <i>hbd</i> , <i>crt</i> , <i>ter</i> , <i>adhE2</i> , <i>fdh</i> KO: <i>adhE</i> , <i>eutE</i> , <i>yqhD</i> , <i>ldhA</i> , <i>ack</i> , <i>pta</i> , <i>pykAF</i> , <i>mdh</i> , <i>frdABCD</i> , <i>yciA</i> , <i>hyc-hyp/fdhF</i>	Batch fermentation of glucose in fermentor with aeration for the first 12 h	20	0.34	0.32	[31]
<i>E. coli</i> MG1655 (pZS-tesBT, pETM-CSA-P)	OE: <i>tesBT</i> , <i>car</i> , <i>sfp</i> , <i>adhE2</i>	Fed-batch fermentation of glucose	2.8	0.10	0.047	[32]
<i>E. coli</i> RB02 Δ <i>yqhD</i> Δ <i>eutE</i> [<i>yqeF</i> + <i>fucO</i> +]	OE: <i>yqeF</i> , <i>fucO</i> KO: <i>yqhD</i> , <i>eutE</i>	Batch fermentation of glucose	13.5	0.27	0.33	[33]

OE: overexpression; KO: knockout, N/A: Not available.

2.1. Metabolic Pathway in *C. acetobutylicum*

Clostridium acetobutylicum is synonymous with industrial ABE fermentation. This microorganism is a strictly anaerobic, spore-forming bacterium known for its biphasic metabolism [34]. This fermentation is characterized by two distinct phases: the acetogenesis phase (*i.e.*, acid production) and the solventogenesis phase (*i.e.*, solvent production). The initial phase is characterized by rapid cell growth, preceding the formation of carboxylic acids, predominantly acetic and butyric acid. Acid excretion decreases the external pH, and these acids induce the biosynthesis of solventogenic enzymes for the subsequent phase of fermentation [35].

In solventogenesis, these acids are reassimilated and serve as co-substrates for solvent production [36]. During the solventogenesis phase, acid production ceases, cell growth halts, and the pH of the medium slightly increases from acid uptake [37]. Carbon and electron flow are redirected toward solvent formation once solventogenesis begins. The transition to solvent production is thought to be an adaptive response by cells to the low pH environment [38]. n-Butanol is majorly produced in this fermentation, with acetone and ethanol as two minor byproducts, where acetone, n-butanol, and ethanol are produced in a 3:6:1 weight ratio.

C. acetobutylicum is a robust microorganism, versatile in its ability to utilize various carbon sources, and adaptable to carbohydrate availability and culture conditions [39]. The acetogenesis and solventogenesis biochemical pathways have been well described, with several studies identifying factors influencing solvent production [3]. *C. acetobutylicum* uses the Embden-Meyerhof-Parnas (EMP) pathway [40] to break hexose sugars into pyruvate, and pentose sugars are metabolized through the pentose phosphate pathway, producing intermediates that enter glycolysis [41]. Historically, n-butanol has been produced from starch and sugar-based feedstocks in industrial ABE fermentation by solventogenic clostridia, including *C. acetobutylicum*, which is limited by low butanol titer (~12 g/L), yield (~0.2 g/g), and productivity (~0.25 g/L·h) [5].

Redox balance plays a major role in *C. acetobutylicum* metabolism and is a phenomenon that is continually being revealed. Recently, Foulquier et al., 2022 characterized the electron pathways used for n-butanol synthesis in *C. acetobutylicum* that were previously unaccounted for [42]. The central metabolism for *C. acetobutylicum* is given in Figure 1. From the EMP pathway, a single mole of glucose is converted to two moles of pyruvate and two moles of NADH, then two reduced ferredoxin and two acetyl-CoA molecules are produced during the decarboxylation of the two moles of pyruvate via pyruvate ferredoxin oxidoreductase (*pfor*). However, during solventogenesis, the production of a single mole of n-butanol from the conversion of two moles of acetyl-CoA produces one mole of reduced ferredoxin generated when crotonyl-CoA is reduced to butyryl-CoA via butyryl-CoA dehydrogenase (BCD). Yet, four moles of NADH are consumed. Additionally, one mole of NADPH is consumed for butyraldehyde reduction via the NADPH-dependent alcohol dehydrogenases [43]. Two moles of NADH are produced from the EMP pathway, and four moles of NADH are consumed for n-butanol production; therefore, the generation of two moles of NADH from the oxidation of two moles of reduced ferredoxin via ferredoxin-NAD⁺ reductase is required. Additionally, the absence of an oxidative pentose-phosphate pathway [44] and the low-level expression of *gapN*, encoding the non-phosphorylating NADPH-producing glyceraldehyde-3-P dehydrogenase, means one mole of NADPH must be produced from the oxidation of reduced ferredoxin via ferredoxin-NADP⁺ reductase. The study showed that ferredoxin-NADP⁺-reductase inactivation considerably lowered butanol production and raised acetone production, whereas overexpression of this gene increased butanol production, resulting in 72% of the theoretical maximum butanol yield from glucose. These findings infer that NAD(P)H flux is a limiting factor to butanol production and demonstrate that electron flux manipulation modulates carbon fluxes.

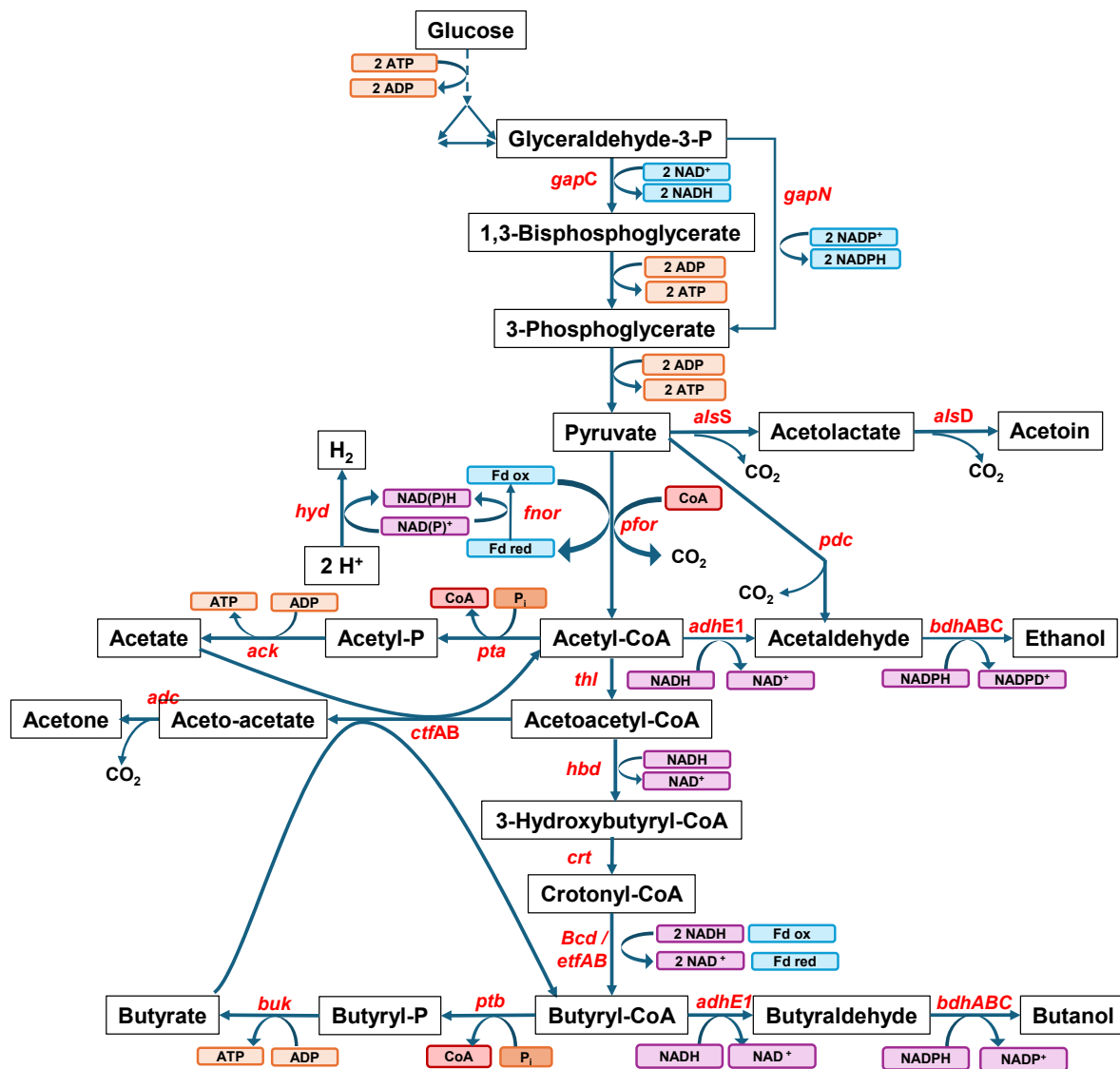


Figure 1. Central metabolism of *Clostridium acetobutylicum*. Genes in the pathways—*ack*: acetate kinase, *adc*: acetoacetate decarboxylase, *adhE1*: aldehyde dehydrogenase, *alsD*: alpha-acetolactate decarboxylase, *alsS*: acetolactate synthase, *bcd*: butyryl-CoA dehydrogenase, *bdh*: butanol dehydrogenase, *buk*: butyrate kinase, *crt*: crotonase, *ctfAB*: CoA-transferase, *etf*: electron transfer flavoprotein, *hbd*: 3-hydroxybutyryl-CoA dehydrogenase, *hyd*: hydrogenase, *fnor*: ferredoxin-NAD(P)⁺ oxidoreductases, *pdc*: pyruvate decarboxylase, *pfor*: pyruvate:ferredoxin oxidoreductase, *pta*: phosphotransacetylase, *ptb*: phosphotransbutyrylase, *thl*: thiolase, *gapC*: NADH-dependent glyceraldehyde-3-phosphate dehydrogenase, *gapN*: nonphosphorylating NADPH-producing glyceraldehyde-3-phosphate dehydrogenase. Fd_{ox} represents oxidized ferredoxin, Fd_{red} represents reduced ferredoxin.

2.2. Metabolic Engineering of *C. acetobutylicum*

Many studies have investigated increasing n-butanol production through the metabolic engineering of *C. acetobutylicum*. Some approaches include mutation and overexpression of thiolase (*thl*) [45], disruption of phosphotransacetylase (*pta*) and butyrate kinase (*buk*) [46], and CoA transferase (*ctfAB*) down-regulation combined with aldehyde/alcohol dehydrogenase (*adhE1*, *adhE2*) overexpression [47]. However, perhaps the most comprehensive work done on the metabolic engineering of *C. acetobutylicum* for enhancing n-butanol production was performed by Nguyen et al., 2018 [17]. Nguyen and his team created a *C. acetobutylicum* strain containing multiple gene deletions and heterologous gene substitutions. Gene knockouts include: *ptb* (phosphotransbutyrylase) and *buk* (butyrate kinase) encoding butyrate biosynthesis from butyryl-CoA; *ldhA*, a gene encoding lactate production; *ctfAB*, a gene encoding acetone formation; and *rexA*, a redox-sensing transcriptional regulator recognized as gene repressor for the C4 formation pathway (*thlA*, *crt-bcd-etfAB-hbd*, and *adhE2*). Gene substitutions $\Delta thlA::atoB$ (acetyl-CoA acetyltransferase from *E. coli*) and $\Delta hbd::hbd1$ (NADPH-dependent 3-hydroxybutyryl-CoA dehydrogenase from *C. kluyveri*) were made to eliminate potential CoA-SH inhibition of the enzyme encoded by *thlA* and to utilize the high NADPH/NADP⁺ ratio driving force. This resulted in a strain which achieved 83% of the theoretical maximum butanol

yield, and a sixfold butanol-to-ethanol ratio increase. However, attempts to eliminate genes *pta* and *ack*, responsible for acetate accumulation, were not successful.

Another pertinent study addressed a central issue described above: the limitation of reducing power in n-butanol production. The disruption of hydrogenase gene *hydA* in *C. acetobutylicum* increased intracellular reducing power and enhanced butanol production with significantly decreased by-products, achieving a high butanol yield of 0.28 g/g and butanol ratio of 84.0% when methyl viologen was added in the fermentation [48]. In addition, researchers introduced a 2,3-butanediol synthesis pathway to mitigate the need for acetone production, which acted as an NADH-compensating module [49]. This study illustrated the role of acetone production in regulating redox balance, where overexpression of a 2,3-butanediol production pathway nearly eliminated acetone production and resulted in a high total alcohol yield of 0.44 g/g glucose. Finally, butanol production was also enhanced through intracellular NADH regeneration in a CdSe-*C. acetobutylicum*_g semi-photosynthetic biohybrid system, which used light-activated cadmium selenide quantum dots (CdSe QDs) to capture electrons from light, resulting in a 45.5% increase in NADH/NAD⁺ ratio compared to *C. acetobutylicum* without CdSe QDs [50]. The photo-fermentation system produced 14.8 g/L butanol at a 0.29 g/g yield from rice straw hydrolysate.

Butanol production has also been increased by overexpressing 6-phosphofructokinase (*pfkA*) and pyruvate kinase (*pykA*), two key genes in the glycolytic pathway, in ABE fermentation due to enhanced butanol tolerance [51].

Strain degeneration due to loss of the pSOL1 megaplasmid containing the *sol* operon genes responsible for solvent production in *C. acetobutylicum* often limits its use in continuous ABE fermentation [52]. This limitation can be mitigated by integrating the *sol* operon genes on the chromosome for stable gene expression for solvent production in a continuous fermentation process [53].

In general, the ABE fermentation is unstable and difficult to control due to clostridia's complex life cycle involving acidogenesis, solventogenesis, and sporulation, which are highly regulated by various kinases, transcription factors, and interlocking signal pathways [54]. Under stress, the cells sporulate and halt their metabolism [55]. Sporulation limits cell's ability to produce butanol and can make clostridial fermentation challenging and prone to failure [56]. Clostridia sporulation is regulated and controlled by Spo0A, which is phosphorylated/dephosphorylated by orphan histidine kinases encoded by *cac3319*, *cac0903*, *cac0323*, and *cac0437* [57–59]. A recent study showed that the knockout of *cac3319* aborted sporulation and enhanced butanol tolerance and production [14]. In contrast, premature sporulation was observed with *cac0437* knockout, which also caused early autolysis, inhibited the transition from acidogenesis to solventogenesis, and resulted in poor butanol production [60]. Finally, a mutant with double knockouts of *cac3319* and *cac0323* gave the highest butanol production of >20 g/L [15]. These studies demonstrated that engineering the orphan histidine kinases can effectively regulate sporulation and increase butanol tolerance and production in *C. acetobutylicum*. Further knockout of *adc* and overexpression of *adhE2* in the mutant with histidine kinase genes (*cac3319* and *cac0323*) knockout decreased acetone production to 0.1 g/L and increased butanol production to 19.7 g/L with a productivity of 0.41 g/L·h and yield of 0.26 g/g [16].

Finally, engineering the accessory gene regulator (Agr) involved in the quorum sensing system, which regulates autoinducing peptides (AIPs) and plays a critical role in intercellular communication, may provide a novel strategy for optimizing cell metabolism and butanol biosynthesis in *Clostridium acetobutylicum* [61].

2.3. Metabolic Pathway in *C. tyrobutyricum*

Acidogenic clostridia contain metabolic pathways to produce acetic and butyric acids, which can be leveraged to produce biofuels, making them novel hosts for n-butanol production. *Clostridium tyrobutyricum* is one such clostridia. Bao et al., 2020 have written a comprehensive review on n-butanol and butyrate production in *C. tyrobutyricum* [11]. It is a super butyrate-producer, which utilizes a butyryl-CoA/acetate CoA transferase (encoded by *cat1*) to re-assimilate acetate for butyrate biosynthesis, unlike other clostridia, which typically use the phosphotransbutyrylase-butyrate kinase (*ptb-bk*) pathway [62]. The central metabolism for *C. tyrobutyricum* engineered for n-butanol production is given in Figure 2. Additionally, *C. tyrobutyricum* does not contain the *ctfAB* operon responsible for production of the acetoacetate intermediate. The absence of this operon essentially eliminates the possibility of acetone production.

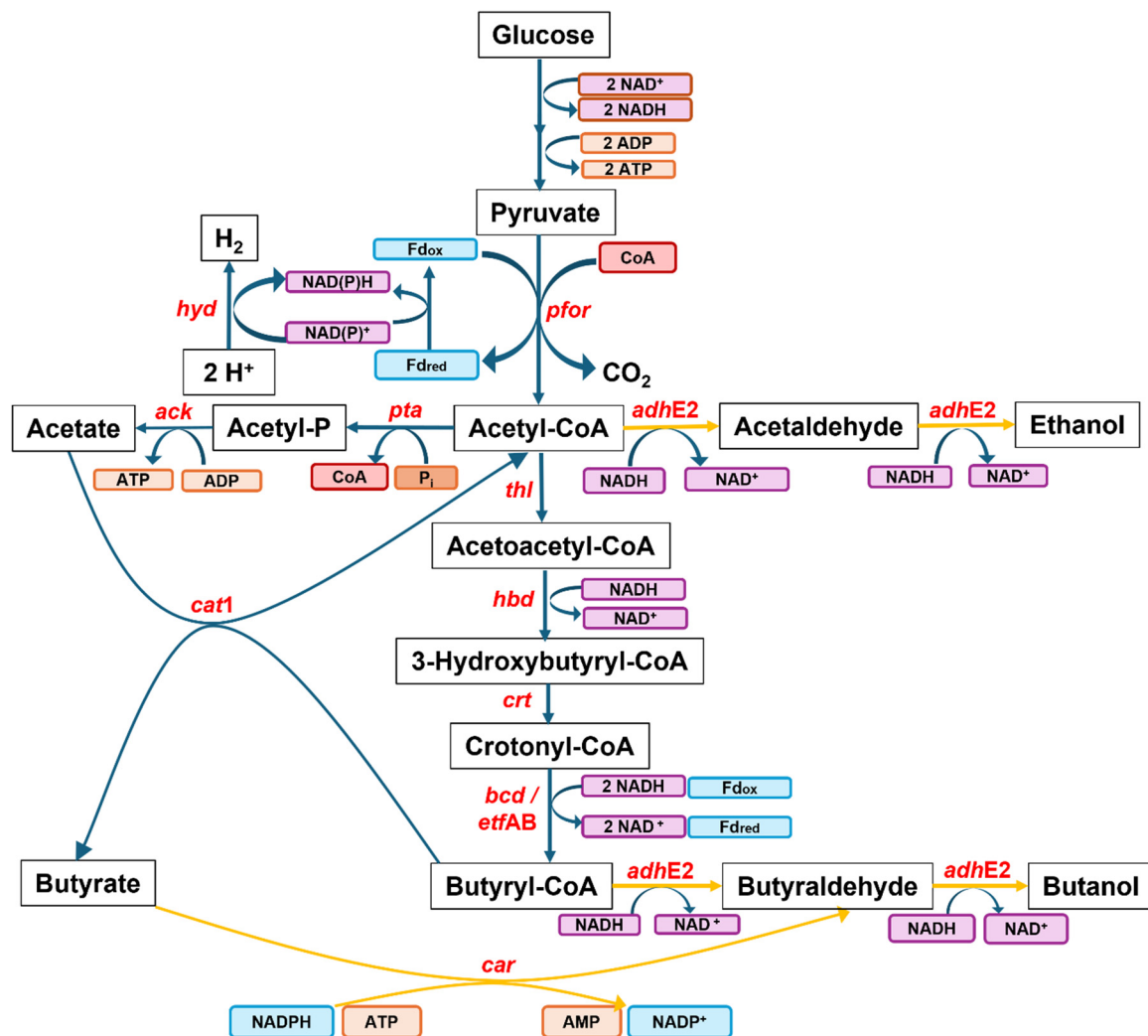


Figure 2. Metabolic pathways in engineered *C. tyrobutyricum* for butyrate and n-butanol production from glucose. The reactions catalyzed by the heterologous enzymes are in orange color. Genes in the pathways—*ack*: acetate kinase; *adhE2*: aldehyde/alcohol dehydrogenase; *adh* alcohol dehydrogenase; *bcd*: butyryl-CoA dehydrogenase; *cat1*: butyryl-CoA/acetate CoA transferase; *car*: carboxylic acid reductase; *crt*: crotonase; *etfAB*: electron transferring flavoprotein; *hbd*: 3-hydroxybutyryl-CoA dehydrogenase; *hyd*: hydrogenase; *pfor*: pyruvate:ferredoxin oxidoreductase; *pta*: phosphotransacetylase; *thl*: thiolase.

A major drawback to biobutanol production in *C. tyrobutyricum* is its narrow substrate spectrum, which is capable of using limited monosaccharides for growth [63]. *C. tyrobutyricum*'s comparatively smaller genome, when compared to other typical butanol-producing clostridia, lacks transporter and catabolism genes for the metabolism of more complex carbohydrates [64]. Efforts have been made to increase the substrate variety of *C. tyrobutyricum*, where heterologous catabolism pathways for various monosaccharides and disaccharides have been effectively implemented into *C. tyrobutyricum* [23–25].

2.4. Engineering Strategies for Increasing n-Butanol Production in *C. tyrobutyricum*

C. tyrobutyricum was first engineered to produce n-butanol in 2011 when Yu et al. overexpressed *adhE2* to reduce butyryl-CoA to butyraldehyde and eventually n-butanol [18]. These researchers achieved a respectable yield of 0.27 g/g glucose. The butanol production in *C. tyrobutyricum* was then increased with the generation of mutant strain *Ack-adhE2* (renamed from Δ *ack-adhE2*), after optimizing the replicon in the conjugative plasmid and utilizing the more reduced substrate mannitol. This work resulted in n-butanol production at 20.5 g/L with a yield of 0.33 g/g [65], yet this fermentation still suffered from high acid production. The group then addressed this by co-overexpressing CoA transferase (encoded by *ctfAB*) from *C. acetobutylicum* to increase n-butanol production via butyrate reassimilation. Butanol productivity and yield increased twofold [22], but it was accompanied by the production of acetone. Zhang et al., 2018 used the native CRISPR-Cas system to knock out *cat1* with genomic insertion of *adhE2* in *C. tyrobutyricum*,

resulting in the mutant strain $\Delta cat1::adhE2$ which produced 26.2 g/L n-butanol, with a yield of 0.23 g/g and negligible butyrate production from glucose [26]. The fermentation with this mutant strain was still accompanied by significant acetate and ethanol production, which limited the butanol yield.

The production of n-butanol in engineered *C. tyrobutyricum* overexpressing *adhE2* follows a very similar pathway to the one used in *C. acetobutylicum*, where it was shown there is an additional need for NAD(P)H. To address this redox imbalance in *C. tyrobutyricum*, researchers have taken a process engineering approach by supplementing methyl viologen (MV) as an electron carrier. This led to more than 80–90% reduction in acetic and butyric acids, increased n-butanol production to 14.5 g/L, and resulted in a yield greater than 0.3 g/g glucose in a *C. tyrobutyricum* $\Delta cat1::adhE2$ fermentation [19]. This increase in butanol production was the result of suppressing hydrogen emissions and increasing NADH availability.

2.5. Metabolic Engineering of *E. coli* for n-Butanol Production

Escherichia coli is another microorganism that has been leveraged for biobutanol production [66]. The Gram-negative, rod-shaped, facultative anaerobic *E. coli* is well understood both physiologically and genetically. *E. coli* can grow on minimal media, utilizes a variety of substrates as carbon sources, possesses a rapid growth rate, can handle a wide range of oxygen concentrations, is tolerant of industrial environments, and is resilient to elevated substrate and product levels, all factors making it an ideal cell factory [67]. Much work has been done on engineering *E. coli* to synthesize butanol. Comprehensive reviews on this topic can be found in recent review articles [12,66,68]. A schematic overview of different metabolic engineering strategies used to produce n-butanol in *E. coli* is given in Figure 3.

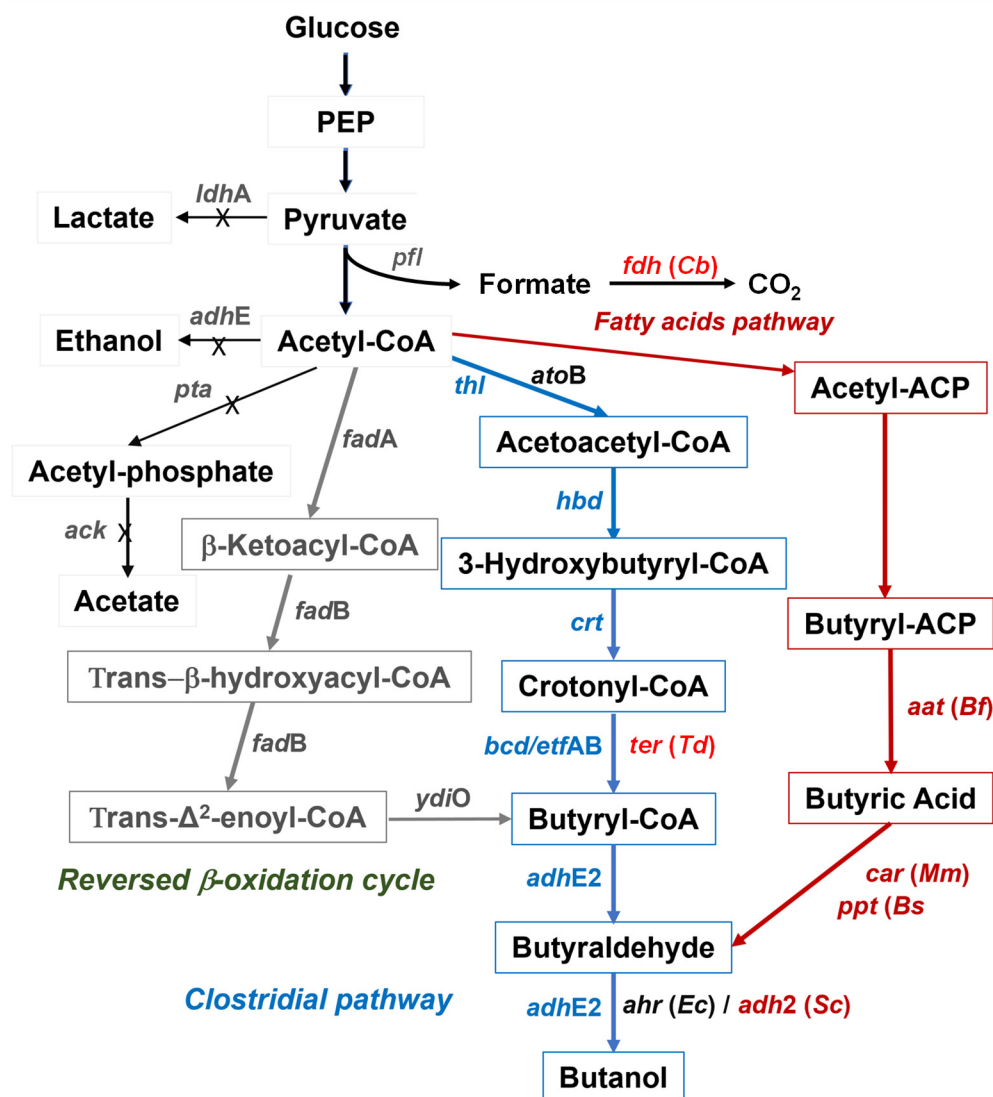


Figure 3. Overview of the different metabolic engineering strategies reported in the literature to produce n-butanol in *Escherichia coli*. *E. coli*'s native genes *ldhA* (lactate dehydrogenase), *adhE* (alcohol dehydrogenase), *ack* (acetate kinase), and *pta* (phosphotransacetylase), are knocked out to eliminate lactate, ethanol and acetate production, whereas a heterologous *fdh* (formate

dehydrogenase) is overexpressed to convert formate produced from pyruvate via pyruvate formate lyase (*pfl*) to CO₂ to increase the intracellular NADH pool. The *Clostridial* n-butanol biosynthesis pathway with associated genes from *Clostridium acetobutylicum* is in blue. *atoB* (acetyl-CoA acetyltransferase) and *ter* (trans-2-enoyl-CoA reductase) are also used as better alternatives to *thl* and *bcd/etfAB*, respectively. The strategy is based on *E. coli*'s native fatty acids pathway and a heterologous pathway with associated genes *aat* (Acyl-ACP thioesterase), *car* (carboxylic acid reductase), *ppt* (phosphopantetheinyl transferase), and *ahr* or *adh2* (aldehyde reductase) leading to butyric acid and then n-butanol is in red. The reversed β -oxidation cycle converting acetyl-CoA to butyryl-CoA via thiolase (*fadA*), hydroxyacyl-CoA dehydrogenase (*fadB*), enoyl-CoA hydratase (*fadB*), enoyl-CoA reductase (*ydiO*) is shown in grey. The sources of the genes are indicated in the parenthesis: *Bf*, *Bacteroides fragilis*; *Bs*, *Bacillus subtilis*; *Cb*, *Candida boidinii*; *Ec*, *Escherichia coli*; *Mm*, *Mycobacterium marinum*; *Td*, *Treponema denticola*. Abbreviation: ACP, acyl-acyl carrier protein.

Ethanol production was the first biofuel process explored in *E. coli*, a compound it naturally produces [69]. Atsumi et al., 2008 made a significant breakthrough by engineering *E. coli* to produce n-butanol [29]. This was made possible by introducing genes (*thl*, *crt*, *hbd*, *bcd-etfAB*, *adhE*) in the biosynthetic n-butanol pathway native to clostridia into the *E. coli* genome. The study reported a butanol titer of 0.55 g/L by further replacing the thiolase (*thl*) with the native *E. coli*'s acetyl-CoA acetyltransferase (*atoB*) and cultivating the cells semi-aerobically in nitrogen-rich media with 2% glycerol supplementation. Butanol production further increased to 15 g/L by replacing *bcd-etfAB* with the *Treponema denticola*'s *ter* encoding trans-2-enoyl-CoA reductase that converted crotonyl-CoA to butyryl-CoA using NADH as a direct reducing equivalent without flavoproteins or ferredoxin [30]. The engineered *E. coli* strain also had its native *pta* encoding phosphate acetyltransferase knockout to direct the acetyl-CoA toward n-butanol, instead of acetate, biosynthesis. In addition, formate dehydrogenase (*fdh*) from *Candida boidinii* was overexpressed to oxidize formate to CO₂ and increase the intracellular NADH driving force, resulting in increased butanol production from glucose at a high yield of ~0.28 g/g or 70% of theoretical in fed-batch fermentation with *in-situ* butanol separation by gas stripping.

Despite the low initial butanol titers, there have been major advancements in n-butanol production in *E. coli*. For example, Dong et al., 2017 developed an *E. coli* strain by completely engineering the chromosome [31]. It could produce butanol without the need of plasmids, antibiotics, or inducing agents. First, the butanol pathway was inserted into the chromosome, and then the host and the butanol pathway were engineered iteratively to stabilize the heterologous butanol pathway in the host. This resulted in *E. coli* strain EB243, with 33 native genes deletions and 5 heterologous genes expressed. The fermentation of strain EB243 gave a titer of 20 g/L butanol, with a yield reaching 83% of the theoretical yield in batch fermentation. This work is an example of how far butanol production in *E. coli* has come.

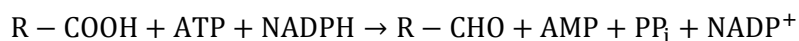
An earlier work also demonstrated n-butanol production from glucose in *E. coli* with engineered reversal of the β -oxidation cycle involving thiolase (*yqeF*, *fadA*), hydroxyacyl-CoA dehydrogenase (*fadB*), enoyl-CoA hydratase (*fadB*), enoyl-CoA reductase (*ydiO*), and alcohol dehydrogenases, achieving a final butanol titer of 13.5 g/L, a yield of 0.27 g/g, and productivity of 0.33 g/L·h in batch fermentation with aeration controlled at 5% saturation of the dissolved oxygen [33].

Most *E. coli* n-butanol production studies focused on engineering the CoA-dependent clostridial pathway, which was anaerobic and required complex nutrients for cell growth. Leveraging the native fatty acids biosynthesis pathway in *E. coli*, an innovative, O₂-tolerant butanol biosynthesis pathway has been constructed in *E. coli* by expressing Acyl-ACP thioesterase (AAT) specific to butyryl-ACP and an oxygen-tolerant carboxylic acid reductase (CAR) [70]. The AAT catalyzed butyric acid production from butyryl-ACP, CAR catalyzed butyraldehyde production from the generated butyric acid, and an *E. coli* aldehyde reductase (*ahr*) then catalyzed the production of n-butanol from butyraldehyde, resulting in butanol production of ~0.3 g/L. More recently, an *E. coli* strain overexpressing CAR from *Mycobacterium marinum*, phosphopantetheinyl transferase (PPT encoded by *sfp*) from *Bacillus subtilis*, alcohol dehydrogenase (*adh2*) from *Saccharomyces cerevisiae* and the thioesterase TesBT was constructed to produce n-butanol from glucose, reaching ~2.9 g/L in a fed-batch fermentation [32]. Although the butanol production via the fatty acids pathway was quite low compared to the clostridial pathway, the novel development here was the use of the heterologous CAR enzyme for the reduction of butyric acid to butyraldehyde. This typically recalcitrant reaction is easily catalyzed by the CAR enzyme.

3. Carboxylic Acid Reductases in Butanol Biosynthesis

3.1. Function and Mechanism of Carboxylic Acid Reductases

Carboxylic acid reductases (CARs) catalyze the reduction of carboxylic acids into their corresponding aldehydes. Classified under EC 1.2.1.30, CARs work by first activating the carboxylate substrate using ATP, then reduce the carboxylate substrate with NADPH serving as the hydride donor [71]. The reaction is given in the equation below.



This metabolic activity was first witnessed in fungi [72], though it was not until the late 1960s that the CAR protein native to the fungus *Neurospora crassa* (NcCAR) was purified and characterized [73]. More recently, there has been a surge in interest in CARs.

CARs are promiscuous enzymes, characterized by a broad substrate range. The substrate specificities largely overlap across CARs with some exceptions, like SrCAR's unique ability to reduce a nitro-substituted benzoic acid derivative [74]. CARs were originally referred to as aryl-aldehyde dehydrogenases (NADP⁺), reflecting their preference for aromatic substrates. Now, it is universally known that CARs are also effective at reducing short to intermediate chain length aliphatic acids.

CARs are composed of three domains: an adenylation domain (A-domain), a transthiolation domain (T-domain), and a reductase domain (R-domain). CARs undergo a post-translational modification, where a conserved serine in the T-domain is activated by a phosphopantetheinyl transferase. The CAR catalytic cycle begins with the activation of the carboxylate substrate. The exact mechanism of aldehyde formation by CAR remains unproven, but current understanding suggests that the process begins when ATP activates the carboxylic acid substrate, creating an AMP-ester. This step is followed by the thiol group of phosphopantetheine catalyzing the transesterification of the AMP-ester, forming a thioester that covalently attaches the acyl group to the CAR. The substrate is then transported from the A-domain to the R-domain, via the T-domain in a “swinging arm” motion [75]. The final step is the reduction of the thioester in the R-domain by NADPH [76]. Although these steps are consistent with the current understanding of the process, further experimental validation is needed, potentially through mutational and kinetic studies like those performed for NRPS domain modifications. DNA alignment of empirically verified CARs has elucidated a CAR signature, composed of four distinct segments containing conserved residues within the A-domain, the phosphopantetheinylated serine region in the T-domain, and three conserved regions in the R-domain. This information is crucial for identifying new CAR sequences.

Researchers have a keen interest in CAR regarding n-butanol production because it can be used to reassimilate by-products for further n-butanol production, and it can be used to engineer novel n-butanol producing pathways. CAR use has been successfully demonstrated in *E. coli* for n-butanol production [32]. As discussed earlier, activity on the substrate butyrate by CAR, isolated from *Mycobacterium marinum* (mmCAR), has already been demonstrated in *E. coli* co-expressing mmCAR, *sfp*, and *adh2*. Until recently, CAR had not yet been utilized in n-butanol production occurring in a clostridia species. There is high potential for its use since it has been demonstrated that it can reduce butyric acid to butyraldehyde. Reduction to butyraldehyde via CAR could increase n-butanol production in *C. tyrobutyricum* since the major byproduct of this n-butanol fermentation is butyric acid. Our most recent study showed that overexpressing mmCAR and *sfp* in the *C. tyrobutyricum* strain overexpressing *adhE2* increased butanol production with reduced butyrate production compared to the strain without co-expressing mmCAR and *sfp* [77].

The promise of selective aldehyde production drives CAR research. However, cofactor demand is a major limitation of CAR utilization in bioproduction. *In vitro*, the high cost of cofactors is inhibitory, and the use of CAR creates a balance between cofactor supply and product selectivity, as the cellular environment reactions often eliminate reactive aldehyde species. To enhance *in-vivo* cascades, one effective approach is to adjust the aldehyde levels to lower concentrations. This can be achieved by fine-tuning the reaction rates of the subsequent transformations. Another method is through channeling, examples include scaffolding proteins or utilizing microcompartments [78].

3.2. Engineering Efforts to Enhance CAR Enzyme Performance

Although some steps of the CAR mechanism remain unclear, there is enough information to achieve kinetic improvements via rational engineering. In work performed by Wang et al., 2022, the catalytic efficiency of a *Mycobacterium smegmatis* originating CAR was increased by engineering the “hinge” mechanism of the CAR enzyme [79]. The team designed a mutagenesis library of the hinge region based on the characteristic construction of CARs and their superfamily. The team was able to achieve a 6.57-fold improvement in catalytic efficiency by introducing the mutations R505I/N506K. The increase in catalytic efficiency is attributable to tight binding of the acyl-AMP complex, as explained by MD simulations.

4. Cofactor Regeneration Systems

4.1. Cofactor Regeneration Overview

NADH availability is a limiting factor in butanol-producing solventogenic clostridia [80]. Strategies employed to overcome this challenge typically fall into one of two categories. The first strategy is metabolic or redox engineering, which involves altering the organism's flux distributions to restore redox balance. The second strategy, known as "Metabolic Process Engineering" (MPE), adjusts the fluxes within the metabolic pathway by manipulating the fermentation environment, without requiring genetic modifications to the organism. For example, the availability of NADH and the resulting butanol production were notably improved when glycerol and mannitol, *i.e.*, more reduced substrates, were used for fermentation [18,81]. Another potential example of MPE that can be utilized is cofactor regeneration.

Cofactors act as enzyme reaction agents, interacting directly with substrates, typically changing only in oxidation state as a driving force for chemical reactions. However, direct stoichiometric supplementation of cofactors in industrial applications is cost-prohibitive. Therefore, there is an essential need to regenerate cofactors. The regeneration of nicotinamide cofactors can be performed using a variety of methods, including enzymatic, chemical, photochemical, and electrochemical approaches [82]. Enzymatic cofactor regeneration typically occurs via one of two methods. The first method employs a single enzyme, utilizing both the reduced and oxidized forms of the cofactor, coupling the synthesis of the desired product with the cofactor regeneration reaction. The second method uses two separate enzymes, one for product synthesis and the other for cofactor regeneration. Yoo et al., 2017 described the attributes of an effective regeneration system, which must meet the following criteria: (1) the process should be practical and cost-effective, (2) the regeneration system must be stable, (3) the separation of the product should be straightforward, and (4) byproduct formation should be minimal [83]. Table 2 shows some examples of cofactor regeneration using various enzymes, including formate dehydrogenase (FDH), glucose dehydrogenase (GDH), and phosphite dehydrogenase (PTDH) in notable biotransformation studies. To date, only FDH and PTDH have been investigated for their potential to increase intracellular NADH levels and butanol production in clostridia.

Table 2. Examples of cofactor regeneration and the enzymes used in notable biotransformation studies.

Cofactor	Cofactor Consuming Enzyme	Cofactor Regenerating Enzyme	Application	Reference
NADH	L-PheDH	NADH oxidase	NADH oxidase from <i>Lactobacillus brevis</i> was used to regenerate NAD ⁺ to increase L-methionine conversion. RESULT: L-Methionine conversion of 100% was achieved in the batch system using NADH oxidase as the regeneration enzyme.	[84]
NADH	LDH	FDH	D-lactate dehydrogenase (LDH) was used to transform 2-oxo-4-phenylbutyric acid (OPBA) to R-HPBA, with concomitant oxidation of NADH to NAD ⁺ , which was regenerated by formate dehydrogenase (FDH) present in whole cells of <i>Candida boidinii</i> . RESULT: R-HPBA production with yeast cells and LDH co-immobilized in a fibrous bed bioreactor operated in fed-batch and repeated batch modes gave stable productivity of 9 g/L·h and yield of 0.95 mol/mol OPBA.	[85]
NADPH	AKR, CAR	FDH	<i>In-vivo</i> and <i>in-vitro</i> enzyme cascades were used to generate 1,4-butanediol and 1,6-hexanediol from 4-hydroxybutanoic and adipic acids. Mutated FDH was utilized for NADPH regeneration. RESULT: <i>in-vitro</i> biotransformations resulted in 90% conversion of 4-HB to 1,4-BDO, and 76% conversion of adipic acid to 1,6-HDO. <i>In-vivo</i> biotransformations resulted in >95% substrate conversion for both 4-HB and adipic acids.	[86]
NADPH	DAPDH	FDH	<i>In-vivo</i> biocatalysis system for the synthesis of D-amino acids from L-amino acids by the co-expression of membrane-associated L-amino acid deaminase from <i>Proteus mirabilis</i> , meso-diaminopimelate dehydrogenases from <i>Symbiobacterium thermophilum</i> , and formate dehydrogenase from <i>Burkholderia stabilis</i> , in recombinant <i>E. coli</i> . RESULT: <i>E. coli</i> whole-cell biocatalyst asymmetrically catalyzed the stereoinversion of L-Phe to D-Phe, with yields >99% in 24 h.	[87]
NADPH	SDH	PTDH	<i>Ralstonia</i> sp. 4506 PTDH was mutated to confer higher selectivity toward NADPH and was utilized in the biotransformation of 3-dehydroshikimate to shikimic acid. RESULT: Thermostable and selective PTDH mutant RsPtxDHARRA showed >95% substrate conversion after 90 min at 45 °C, whereas wild-type RsPtxD reaction ceased after 30 min.	[88]
NCD	D-LDH	FDH	FDH from <i>Pseudomonas</i> sp. 101 was engineered to regenerate nicotinamide cytosine dinucleotide (NCD) selectively. RESULT: Most active mutants reached a cofactor preference switch from NAD to NCD by 3700-fold. FDH was coupled with NCD-dependent D-lactate dehydrogenase, where 5.0 mM formate was exhausted in 30 min with an equal amount of D-lactate generation, displaying a perfect stoichiometry.	[89]
NMNH	multiple	GDH	<i>In-vitro</i> transformations utilizing a GDH engineered for NMNH specificity and NMNH supplementation, combined with one of three different reductases: XenA, OYE3, or NfsB. <i>In-vivo</i> biotransformations with multiple reductases co-expressed simultaneously along with a GDH for cofactor regeneration. RESULT: Transformations using these enzyme combinations with NMNH supplementations resulted in conversions of >99%, >99%, and >92% on their respective substrates. <i>In-vivo</i> biotransformations showed the ability to selectively produce levodione based on the GDH mutant, due to XenA and GDH specificity toward NMNH.	[90]
ATP	CAR	PPK12	PPK12 was evaluated for ATP recycling in car-catalyzed 4-methoxybenzoic acid reduction. RESULT: reaction reached >99% conversion required 20% less substrate than the control and half the duration	[91]

4.2. Formate Dehydrogenase

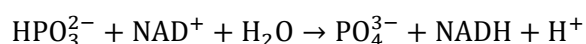
Formate dehydrogenase (FDH, E.C. 1.2.1.2) catalyzes the oxidation of formate to carbon dioxide by reducing NAD^+ to NADH [92]. The innocuous carbon dioxide produced can be released safely into the environment. Formate is inexpensive and non-toxic to most enzymes, serving as a suitable substrate. The irreversibility of this reaction offers a significant advantage for commercial applications. Formate dehydrogenase is the most developed enzyme for cofactor regeneration and is commercially available. Several studies demonstrate the utility of FDH in *Clostridium*. For instance, the NAD^+ -dependent formate dehydrogenase FDH1 gene from *Candida boidinii* was overexpressed for NADH regeneration in *Clostridium paraputrificum*, which increased hydrogen yield by 59% compared with the control [93]. Similarly, the expression of the heterologous formate dehydrogenase in *Clostridium ljungdahlii* also facilitated the regeneration of NADH and increased its intracellular concentration from 2.93 to 12.64 μM per g DCW [94]. In *Clostridium tyrobutyricum*, introducing a heterologous formate dehydrogenase with the bifunctional aldehyde/alcohol dehydrogenase increased butanol production from glucose to 12.3 g/L, an increase of ~18% compared to the control strain without expressing the FDH [95].

4.3. Glucose Dehydrogenase

Glucose dehydrogenase is another enzyme being developed for cofactor regeneration. GDH (E.C. 1.1.1.47) catalyzes the β -D-glucose to β -D-glucono-1,5-lactone oxidation reaction while concurrently reduces the cofactor NAD(P)^+ to NAD(P)H . However, it also catalyzes NAD^+ to NADH to a lesser extent [96]. There are several examples of GDH used for cofactor regeneration. For example, the GDH from *Bacillus* sp. [97] was used to regenerate NADH in the synthesis of L-6-hydroxynorleucine from 2-keto-6-hydroxyhexanoic acid via glutamate dehydrogenase. GDH from *Bacillus* sp. was again used in NADH regeneration in the production of L-carnitine from 3-dehydrocarnitine via L-carnitine dehydrogenase [98].

4.4. Phosphite Dehydrogenase

A third, and more recently discovered enzyme used in cofactor regeneration is phosphite dehydrogenase (PTDH, E.C. 1.20.1.1), an NAD^+ dependent enzyme oxidizing inorganic phosphite (hydrogen phosphonate) to phosphate [99,100]. This reaction is given in the equation below [101]:



This reaction is unique in that there is a hydride donor to hydroxide acceptor phosphoryl transfer, a rare occurrence. During the phosphorus atom oxidation process, the phosphorus atom's oxidation state changes from +3 to +5, resulting in a -0.65V redox potential for the phosphite/phosphate couple. This makes the phosphite oxidation reaction essentially irreversible, a major advantage of using PTDH for cofactor regeneration.

The most extensively studied PTDH enzyme originates from the microorganism *Pseudomonas stutzeri* WM88. This enzyme was first described by White and Metcalf, 2002 to be effective at oxidizing reduced phosphorus compounds [100]. This strain was initially detected by using a selection medium consisting solely of hypophosphite or phosphite as the only phosphorus sources. It was revealed that hypophosphite is oxidized to phosphate in a two-step process, with phosphite as an intermediate. The *htx* and *ptx* loci are the responsible gene clusters, with the gene product PtxD (PTDH) being the agent responsible for catalyzing the phosphite oxidation [99]. The expression of the PtxD gene could be induced by phosphate starvation [102].

The PTDH enzyme shares a resemblance to D-hydroxy acid dehydrogenases, while reactions catalyzed by PTDH are notably different. It shares the key active site residues, including Arg237, Glu266, and His292 required for binding and/or catalysis [101]. His292 is likely the active site base responsible for deprotonating the water nucleophile, though it is possible that it serves as a nucleophilic catalyst. Other critical residues and their role include: Arg237 (phosphite binding), Glu266 (positioning of residues His292 and Arg237), Lys76 (stabilization of the dianionic phosphite substrate), and Glu175 and Ala176 (cofactor binding and cofactor specificity). It is suspected that the rate limiting step is the hydride transfer from the phosphite to the nicotinamide cofactor [103]. PTDH has a strict specificity for NAD^+ , however, using rational design methods, it has been engineered to reduce NADP^+ . Using site directed mutagenesis researchers were able to eliminate steric and repulsive constraints for NADP binding [104]. Replacing the two residues Glu175 and Ala176 with Ala175 and Arg176, respectively, produced PTDH mutants with enhanced cofactor promiscuity. All mutants created demonstrated drastically improved catalytic efficiency for both cofactors, with the double mutant showing the best kinetic performance. The G175A/A176R mutant exhibited a 3.6-fold increase in

catalytic efficiency for NAD^+ and a 1000-fold increase for NADP^+ . The preference shifted from a 100-fold bias toward NAD^+ in the wild-type enzyme to a 3-fold preference for NADP^+ in the double mutant. Co-expressing the mutated PTDH with *adhE2* in *C. tyrobutyricum* showed dramatically increased butanol and reduced butyrate and acetate production compared to the strain without co-expressing the PTDH [77].

5. Protein Engineering of Aldehyde/Alcohol Dehydrogenase for Enhanced Butanol Production

5.1. AAD Overview

Many studies have focused on metabolic engineering to increase n-butanol production, whereas utilizing protein engineering to increase the metabolic flux of a particular reaction is typically an afterthought. Various studies have started to consider this, with appealing results. Often, good enzyme candidates for protein engineering have high substrate promiscuity, low turnover, or strict selectivity for a limited or unavailable cofactor. One enzyme in the *C. acetobutylicum* metabolic pathway that checks many of these boxes is the aldehyde/alcohol dehydrogenase (AAD) encoded by *adhE2*.

In continuous culture, *C. acetobutylicum* is typically characterized as having two distinct metabolic states [105], acidogenesis (production of acetate and butyrate) when grown near pH seven on glucose, and solventogenesis (production of acetone, butanol, and ethanol) when grown at low pH on glucose. However, a third, distinct, metabolic state exists; alcohologenesis (formation of butanol and ethanol but not acetone) occurs when grown at near pH seven under conditions of high nicotinamide cofactor availability. It was discovered that in methyl viologen induced alcohologenic cultures, the genes involved in solventogenesis for butanol biosynthesis were not transcribed, leading to the discovery of *adhE2* [106].

AdhE2 encodes a bifunctional, aldehyde/alcohol dehydrogenase with strict NADH dependence responsible for butanol production in alcohologenic *C. acetobutylicum* cultures. The aldehyde dehydrogenase (ALDH) domain is located at the N terminal of the gene, where a conserved region is located as characteristic of an aldehyde dehydrogenase catalytic center [107]. The domain also contains the two nucleotide binding sites, GVVXG and GCGXXG, sites conserved in ALDH proteins [106]. The alcohol dehydrogenase (ADH) domain is located at the C terminal of the gene, where it possesses a GXGXXVXXA conserved sequence, involved in coenzyme binding. The ADH domain also contains the three histidines of the iron-binding motif, a highly conserved iron-binding site.

A recent study [108] elucidated the roles of two AADs, *adhE1* and *adhE2*, in the primary metabolism of *C. acetobutylicum*. The study revealed that, regarding n-butanol formation, *adhE1* plays the prominent role during solventogenesis, whereas *adhE2* plays a prominent role during acidogenesis and alcohologenesis.

Little research has been done on the specific structure and catalytic mechanism of the *C. acetobutylicum adhE2* enzyme, but it shares much sequence similarity with the *E. coli adhE* gene. We can infer features of the *adhE2* enzyme based on this similarity. The *adhE* enzyme from *E. coli* is a bifunctional aldehyde/alcohol dehydrogenase highly conserved in a variety of organisms, with *adhE* playing a major role in anaerobic conditions. The reduction of acetyl-CoA to ethanol is a two-step reaction: acetyl-CoA is converted to acetaldehyde in the N-terminal aldehyde dehydrogenase (ALDH) domain, and acetaldehyde is converted to ethanol in the C-terminal alcohol dehydrogenase (ADH) domain, with both domains linked together through a short linker. The close proximity of these two domains to each other likely indicates there is substrate channeling to improve the ethanol production rate [109].

5.2. Engineering AADs for Improved Catalytic Efficiency

Several studies have implied that the final steps in the metabolic pathway of n-butanol production, butyryl-CoA reduction and butyraldehyde reduction, can be rate limiting. Increasing the turnover of the *adhE2* enzyme can relieve this bottleneck and increase butanol production. Increasing butanol selectivity is also on the wish-list. These mutation studies have not yet been performed on *adhE2*, but studies have been done on similar enzymes, such as *adhE1* from *C. acetobutylicum* and alcohol dehydrogenase from *Zymomonas mobilis*. Rellos et al., 1997 studied the substrate specificity of *Z. mobilis* alcohol dehydrogenase-2 enzyme using random mutagenesis and discovered that mutations to residues 161, and 155 and 165 enabled the enzyme to accommodate longer-chain alcohols [110]. Specifically, the researchers created the double point mutant A161I, R165H with a $V_{\max}$ that was 135-fold higher than the wild type. This study is a demonstration of the ability to increase enzyme turnover by active-site mutation. Another great example of increasing activity through mutation is the work performed by Cho et al., 2019, who used site-saturation mutagenesis to mutate residues close to the active site of the alcohol dehydrogenase domain of *adhE1* from *C. acetobutylicum* [111]. In their work, two mutations, F716L and N655H, were found to drastically increase the butanol: ethanol ratios by 5.8-

fold and 5.3-fold, respectively, when compared to the wild-type enzyme. Table 3 lists some of the notable studies in engineering AAD for enhanced butanol production.

Table 3. Notable examples of engineering alcohol dehydrogenase to increase production.

Dehydrogenase Enzyme	Mutation(s)	Description	Reference
ZADH2	A161I, R165H; D38G	Mutations to residues 161 and 165 increase activity with longer-chain alcohols, mutations to residue 38 increases NADPH activity	[110]
AdhE1-AAD	M619G, N655H, F716L	mutations increased the butanol:ethanol ratio from fermentation for <i>C. acetobutylicum</i> M5 strain by 2.22-, 5.3-, and 5.8-fold	[111]
AdhE1-AAD	D485G	The D485G mutation increased butanol yield by 4%	[46]
AdhE-AAD	D494G	D494G mutation made to <i>C. thermocellum</i> adhE-AAD. Used to increase NADPH cofactor specificity. Fermentation using this AAD resulted in 1.7-fold increase in butanol yield when compared to the wild-type strain.	[112]
AdhD	G211C, G211InsC	Mutations were made to the AdhD gene from <i>Pyrococcus furiosus</i> to increase NADPH activity. Both mutations, the G211C point mutation and the Cysteine insertion, resulted in an activity increase of over 90-fold.	[113]
TbSADH	H42T	The <i>Thermoanaerobacter brockii</i> ADH was engineered to improve the proton transfer process. The H42T mutant showed a >99% conversion.	[114]

6. Challenges and Future Directions

6.1. Challenges Associated with AAD Engineering

The metabolic and protein design strategies described above are vastly different, and each one has its own challenges and limitations. Protein engineering of the AAD enzyme by either rational design or directed evolution strategies shows great promise for increasing n-butanol production. One challenge that has become apparent is that as the enzyme is mutated to increase the turnover of the larger butyraldehyde substrate, it typically also increases acetaldehyde turnover. What is sought is the increase in butyraldehyde turnover and a decrease in acetaldehyde turnover to increase the selectivity of the enzyme meanwhile maintaining or increasing the catalytic efficiency. This is a classic “multi-state” design problem. The use of directed evolution in this case is straightforward, one directs the evolution of this enzyme to increase butanol production meanwhile maintaining or increasing selectivity. In the past, multi-state design problems were challenging to address when using rational design approaches. There are plenty of examples in the literature where rational design has been used to address multi-state design problems with good results. For example, Schmitz et al., 2017 developed a multi-state framework (MSF) in the Rosetta Macromolecular Modeling Suite that enables the implementation of Rosetta’s single-state protocols in a multi-state environment [115]. Utilizing MSF, the researchers demonstrated a 15% higher performance using MSF than single-state design on a ligand-binding benchmark. The researchers then used this protocol to design nine *de-novo*, retro-aldolases, with all variants displaying measurable catalytic activity, thus illustrating the efficacy of the concept. By utilizing these new design tools to engineer AAD for increased selectivity, it may be possible to achieve higher butanol titers and yields than using single-state rational design alone.

6.2. Challenges Associated with PTDH Usage

An additional challenge, this time presented from PTDH use for cofactor regeneration, is the fact that a general increase in the redox ratio of nicotinamide cofactors may be detrimental to bioproduction. This could be due to cofactor transcriptional regulation, overproduction of side-products, or substrate inhibition of an accompanying enzyme in the cascade. There has been work undertaken to address these limitations, and the answer may be the use of orthogonal cofactors. Orthogonal cofactors, also known as noncanonical cofactor biomimetics (NCBs), are moieties that can function as cofactors in enzymatic catalysis yet are structurally distinguishable from natural nicotinamide cofactors [116]. The use of NCBs offers significant advantages, including improved stability [117], and the ability to reduce interference between modified pathways and the host’s natural metabolic processes [90,118]. A commonly used NCB is nicotinamide mononucleotide NMN(H). NMN(H) is identical to the nicotinamide cofactors but lacks the adenosine binding handle used in enzyme recognition. Black et al., 2020 developed a glucose dehydrogenase with a 107-fold shift in cofactor specificity favoring NMN⁺ over NADP⁺ [90]. The researchers then demonstrated the ability of the system to drive diverse redox reactions *in vitro*, with a high total turnover number (~39,000). Finally, the toolkit was completed

by establishing Nox Ortho [119], a reduced NMN^+ (NMNH)-specific oxidase. When GDH ortho and Nox ortho are used together the enzymes can modulate $\text{NMNH}:\text{NMN}^+$ ratio. The team showed the NMN(H) cofactor pool could be manipulated to achieve a chosen redox ratio at will, ranging from 0.07 to 70 for $\text{NMNH}:\text{NMN}^+$. Additionally, this NMN(H) cofactor pool is dissociated from both nicotinamide cofactor redox ratios. A (S)-specific butanediol (BDO) dehydrogenase was then designed to utilize NMN(H). Using the orthogonal, NMN(H) driving force, chiral-pure 2,3-BDO was produced to near completion, unaffected by NAD(P)/H reduction potentials *in vitro* or *in vivo*.

6.3. Challenges Associated with CAR Usage

For CAR, a major limitation of the use of this enzyme is its need for NADPH and ATP cofactor. Again, it may be possible to address both using cofactor regeneration, although the regeneration of cofactor ATP has not been as straightforward. ATP regenerating methods starting from ADP are well-established, but these methods have disadvantages such as price, instability, and phosphate donor availability [120]. For these reasons more attention has been given to the polyphosphate kinase (PPK)/polyphosphate (polyP) ATP regeneration system. It was thought that there were only two PPK2 classes, PPK2-I and PPK2-II, where PPK2-II would generate nucleoside 5'-diphosphates from nucleoside 5'-monophosphates and polyphosphate consumption and PPK2-I would generate nucleoside 5'-triphosphates from the corresponding nucleoside 5'-diphosphates and polyphosphate consumption. But the recently discovered PPK2-III shows great promise because it is bifunctional, capable of forming both nucleoside-5' diphosphates and nucleoside 5'-triphosphates, reducing the need to overexpress two separate PPK2 enzymes for ATP regeneration [121]. The PPK2 subfamilies and preferred catalyzed reaction(s) related to ATP regeneration are given in Figure 4. The promise of the PPK2-III class of enzyme is highlighted in the work performed by Tavanti et al., who screened a panel of 82 putative PPK2-III enzymes to discover the gene PPK12, isolated from an unclassified *Erysipelotrichaceae* bacterium [91]. The researchers used the PPK12 enzyme for polyP-based ATP recycling to power aldehyde production via a CAR catalyzed reaction. This resulted in several gram scales, cell free aldehyde synthesis, needing substantially less enzyme compared to the ajPAP process, a PPK2-II enzyme of high promise. This work emphasizes the feasibility of ATP regeneration using a PPK2-II enzyme for carboxylic acid reduction via CAR.

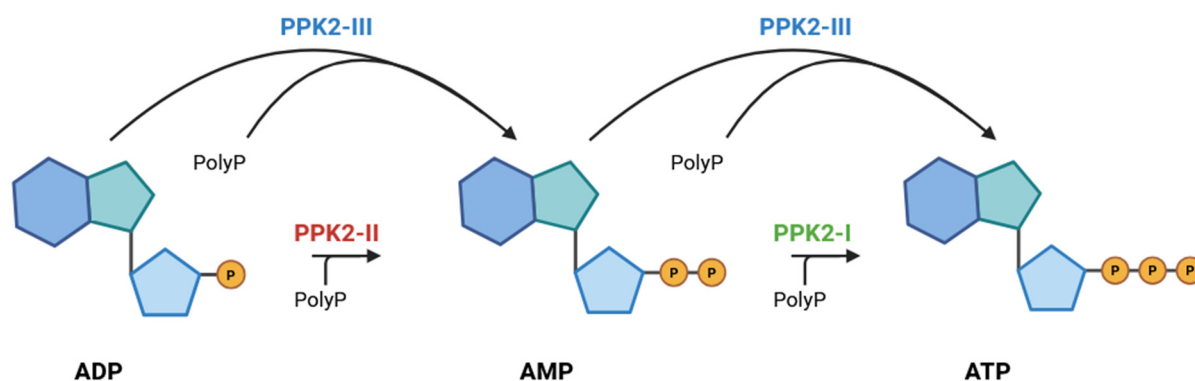


Figure 4. PPK2 subfamilies and preferred catalyzed reaction(s) related to ATP regeneration.

It is noted that ferredoxin-associated aldehyde oxidoreductase (AOR) can also reduce carboxylic acids to aldehydes under anaerobic conditions. Autotrophic acetogens such as *C. ljungdahlii* and *C. autoethanogenum* produce ethanol from acetate via AOR by reducing acetate to acetaldehyde and then to ethanol via ADH [122,123]. *C. carboxidivorans* produces ethanol and n-butanol via the AOR-ADH pathway under autotrophic growth and the ALD-ADH pathway under heterotrophic conditions. AOR plays a critical role in ATP generation under energy-limited conditions and ethanol/acetate formation in acetogens during gas fermentation [124]. Also, exogenous acetate can promote the expression of AOR and ethanol production from acetate [125]. However, to maintain redox and energy balance in response to different fermentation conditions, AOR catalyzes the reaction from acetate to acetaldehyde in CO fermentation but catalyzes the reverse reaction in CO_2/H_2 fermentation [126]. AOR's function also depends on pH, reducing acetate/butyrate to aldehydes with reduced ferredoxin at pH 4.0–5.0 while oxidizing aldehydes to acetate/butyrate with oxidized ferredoxin at pH >5.0 [127]. Overexpressing *aor* or *adhE2* in *C. carboxidivorans* increased its alcohol production from glucose, demonstrating the potential of increasing alcohol production by overexpressing these genes in two different alcohol production pathways [128]. An earlier study has also shown that

the resting cells of *C. formicoaceticum* converted carboxylates to corresponding alcohols in the presence of CO or formate and an electron mediator [129], confirming the presence of ALD, ADH, and AOR activities in *C. formicoaceticum* and their ability to convert acetate to ethanol and butyrate to n-butanol [127]. However, AOR uses reduced ferredoxin (Fd_{red}) as the cofactor, which has a low redox potential (ca. -420 mV at pH 7.0), in transferring the electron to carboxylate. The regeneration of Fd_{red} requires a highly reduced electron donor, such as the reduced methyl viologen ($E_h = -450$ mV), which may be too expensive to use in industrial fermentation.

7. Conclusions and Prospects

Biobutanol production is limited by available reducing equivalents (NADH, NADPH, and Fd_{red}) and the coproduction of ethanol, acetate, and butyrate. Metabolic and redox engineering approaches have been used to increase butanol production, with the butanol yield exceeding 80% of the theoretical yield (0.41 g/g glucose). However, about one third of the substrate carbon in glucose is lost as CO_2 in glycolysis through the EMP pathway, which not only limits product yield but also causes environmental concern due to the release of CO_2 , a major greenhouse gas. It is thus desirable to capture and utilize the fermentation-produced gases (CO_2 and H_2) for butanol production. There are carboxydutrophic acetogens that can convert CO_2 to acetyl-CoA with H_2 or CO as the energy source and electron donor via the Wood–Ljungdahl pathway [130]. Some mixotrophic acetogens can concurrently utilize sugars and gases (CO_2 and H_2) for acetate production with a >95% (w/w) yield without losing much carbon to cell biomass or CO_2 [20,131–133]. However, gas fermentation is limited by the extremely low solubility of CO_2 and H_2 in water. To overcome this problem, CO_2 can be converted to formate first via an electrochemical reduction process [134] and then to acetate using a homoacetogen such as *C. formicoaceticum*. Since *C. tyrobutyricum* can convert glucose and acetate as co-substrates to butyrate [135], it is possible to convert all glucose carbon to butyrate by reassimilating the CO_2 released in glycolysis through formate and acetate by using *C. formicoaceticum*. If *C. tyrobutyricum* expressing *adhE2* can be further engineered to efficiently utilize CAR or AOR to convert butyrate to butyraldehyde and then to butanol, it is possible to attain an overall butanol yield of 0.6 g/g glucose without any CO_2 emission. This may require the use of orthogonal cofactors such as NMN(H) to provide additional reducing equivalents needed for butanol biosynthesis. Together with the engineered AAD (encoded by the *adhE2*) with a high selectivity for butanol over ethanol, a novel butanol producing strain with close to 100% carbon recovery can be developed for sustainable production of biobutanol for fuel and industrial applications.

Author Contributions

C.D.M.: Conceptualization, Writing-original draft, Visualization; Q.W.: Writing-review & editing; G.W.: Writing-review & editing; S.-T.Y.: Conceptualization, Writing-review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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