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Review

Integrated Catalytic Upgrading of Biomass-Derived Alcohols for Advanced Biofuel Production

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Abstract: Sustainable biofuel production is necessary to meet the daunting challenge of "fueling" growing economies with a significantly reduced carbon footprint. Although its higher oxygen content often hinders the direct conversion of lignocellulosic biomass (LCB) into energy-dense biofuels, microbial biofuel production from LCB still has potential. The production of primary alcohols by acetone–butanol–ethanol (ABE) fermentation has been practiced for more than a century to attain near-theoretical maximum. However, ABE produced conventionally by native microorganisms is not equivalent to fossil fuel-based aviation fuels in terms of energy density, volatility, and cost-efficiency. Various strategies have been adapted for the microbial synthesis of advanced fuels from renewable feedstock with the advancements in genetic engineering. Yet, the presence of inhibitors and the inefficiency of microbes to utilize or transport the sugar mixtures from LCB often impede titer and yield. However, ABE mixtures can act as platform chemicals to synthesize high-value biofuels by biocatalytic or chemo-catalytic applications. Chemical catalysts, in particular, are used to produce higher alcohols ranging from 3-carbon to 20-carbon fuels from the ABE fermentation mixture. This article reviews the recent trends in the production of higher biofuels from ABE mixtures using biological and chemical catalysts. Focus is placed on genomic and metabolic engineering strategies implemented to upgrade microbes for higher biofuel production via the fermentation of renewable feedstocks. This paper also summarizes the advancements in the chemical conversion route of an ABE fermentation mixture into higher biofuels. Finally, the review provides insights into future research toward commercializing renewable and sustainable higher biofuels and chemicals.

Keywords: ABE fermentation; higher biofuels; metabolic engineering; chemical catalysts

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1. Introduction

The daunting task of meeting more than 80% of the world's energy needs has exhausted the possibility of the continued use of non-renewable petroleum feedstock [1,2], especially as these fossil fuel reserves are under the control of a few countries, resulting in many political struggles. Years of efforts by multinational organizations have not yet been completely fruitful; for instance, the second commitment period of the Kyoto Protocol was adopted in 1997 and ended in 2020 [3], and yet, a simple unified solution to obtain sustainable and cleaner fuels has not been achieved. Alternative energy sources such as wind and solar energy have been used in suitable regions. However, biofuels, made typically from lignocellulosic biomass (LCB), are necessary to decarbonise those parts of the economy, such as aviation, with no alternative energy sources such as electrification [4]. In this aspect, biofuels have immense potential, as their sustainable production with reduced cost and carbon footprint can be theoretically achieved by harnessing renewable waste LCB [5]. Microbial biofuels can be generated via their naturally existing metabolic pathways—sugar, fatty acid, and isoprenoid pathways [6]. However, native microorganisms are inefficient in

producing advanced biofuels that can be used as drop-in fuels or substitutes for aviation or diesel fuels. With the advent of modern genome engineering applications, coupled with those in product recovery, the production of primary alcohols, viz., ethanol and butanol from acetone–butanol–ethanol (ABE) fermentation, can be achieved at near-maximal theoretical yield (Figure 1) [7,8]. Table 1 comprehensively compares the fuel properties of ABE fuels and conventional fuels. However, the toxicity of substrates, inhibitors, and often the end-product itself, diminishes the productivity and titer of advanced biofuels. Therefore, using genome-engineering strategies, there is a need to enhance feedstock utilization and improve inhibitor tolerance for advanced biofuel production through microbial fermentation. Integrating chemical alkylation with ABE fermentation could be a promising approach to convert ABE fermentation broth into long-chain fuels (C_5–C_{20} biofuels) sustainably [9]. Hence, it is necessary to optimize and scale up the production of advanced biofuels from LCB using a combination of genetic and metabolic engineering, bioprocess engineering, downstream processing, and catalytic approaches, while addressing the challenges of toxicity and inhibitor tolerance.

This review presents an overview of the recent developments in integrating higher biofuel production with a catalytic approach. The implications of genome engineering strategies to enhance feedstock utilization and improve inhibitor tolerance for advanced biofuel production through microbial fermentation are discussed in detail. Furthermore, the integrated catalytic approaches involved in the sustained conversion of ABE fermentation broth into long-chain fuels are documented.

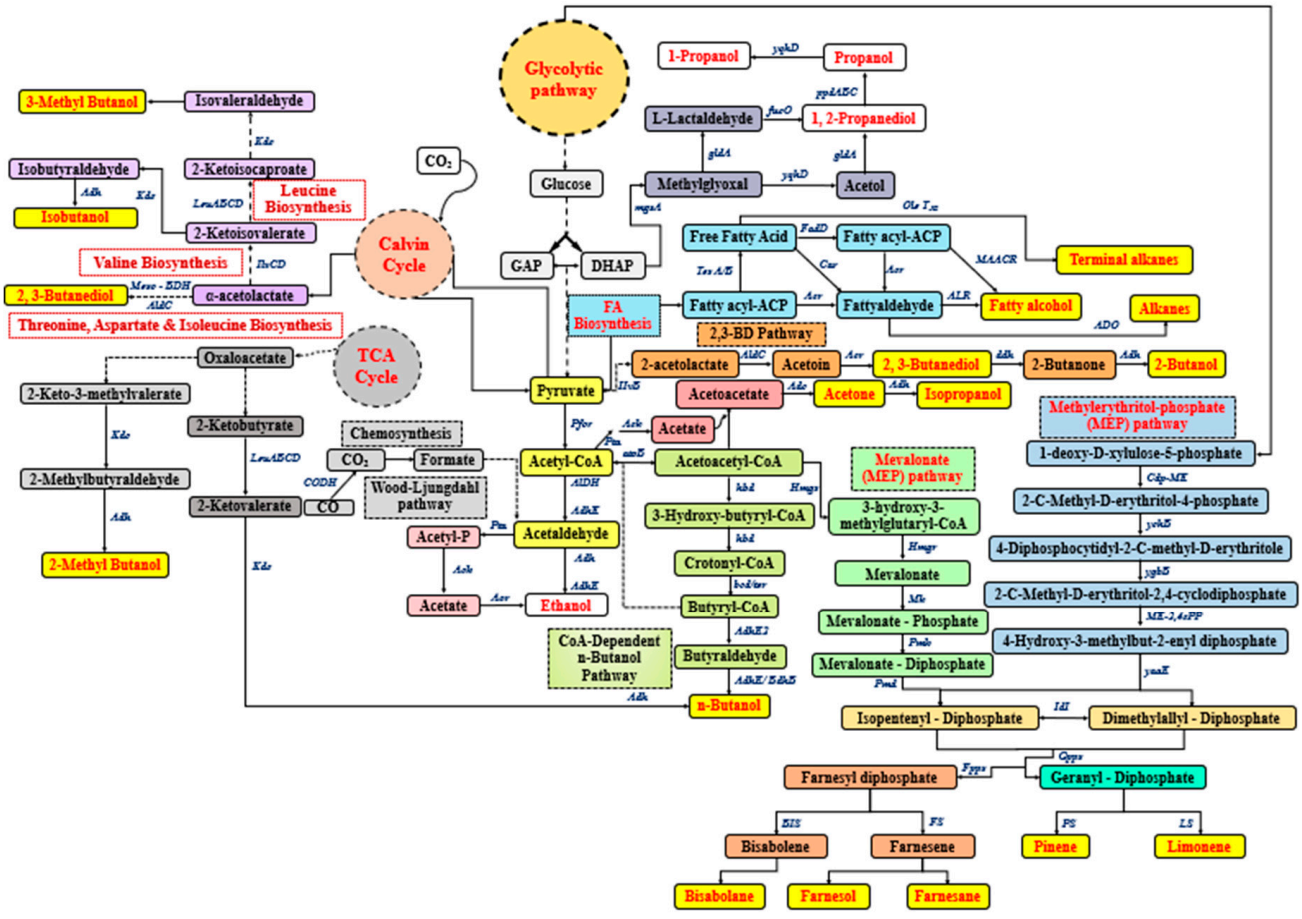

Figure 1. Schematic representation of microbial metabolic (native and engineered) pathways involved in production of advanced fuel precursors.

Table 1. Comparative fuel properties of the conventional fuels and ABE.

Product	Chemical Formula	Cetane Number	Octane Number	Density (g/mL) [a]	Viscosity (mm^2/s) [b]	Energy Density (MJ/l)	Reference
Gasoline	C_4–C_{12}	0–10	88–90	0.77	0.49	31.0–33.2	Veza et al. [10]
Diesel	C_{12}–C_{25}	40–55	20–30	0.82–0.86	1.9–4.1	35.0–36.7	Wu et al. [11]
Acetone	C_3H_6O	NA	117	0.79	0.35	23.40	Lapuerta et al. [12]
Butanol	C_4H_9OH	25	96	0.81	2.63	26.90	Wallner et al. [13]
Ethanol	C_2H_5OH	5–8	100	0.80	1.08	21.30	Veloo et al. [14]
ABE (3:6:1)	$C_{3.5}H_{8.4}O$	NA	102.7	0.80	1.79	25.29	Zhang et al. [15]

[a] Density at 288 K; [b] Viscosity at 413 K.

2. Bio-Catalytic Generation of Advanced Biofuels

Noteworthy discoveries in genetic engineering strategies for the bio-catalytic generation of advanced biofuels, categorized based on the type of fuel, have been highlighted in Table 2. A detailed and comprehensive analysis is provided in the subsequent section.

Table 2. Biocatalytic generation of advanced biofuels using genetic engineering strategies.

Target Compound	Organism	Substrate	Genetic Engineering Strategy	Production Strategy		Reference
				Yield (g/g)	Titer (g/L)	
Higher alcohols						
Butanol	*Escherichia coli*	Glucose + butyrate	Overexpression of *thL*, *hbd*, *crt*, *bcd-eftB-eftA*, *adhe1/adhe* from *Clostridium acetobutylicum*	1.20 [a]	NA	Inui et al. [16]
1-butanol	*Escherichia coli*	Glucose	Overexpression of *ldhA*, *frdABCD*, *fdh*, *adhE*, and *ackA*	0.25 [a]	10 [f]	Wen and Shen [17]
1-butanol and 1-propanol	*Escherichia coli*	Glucose	Overexpression of *kivd*, *adh2*, and *ilvA*, *leu-ABCD*, *thrA*, and *fbrBC*, and elimination of *metA* and *tdh*	NA	2.00 (1:1)	Shen and Laio [18]
Butanol	*Clostridium acetobutylicum* strain PJC4BK		Inactivation of *buk*	NA	11.7–16.7	Harris et al. [19]
Butanol	*Clostridium acetobutylicum*	Glucose	Disruption of *pta* and *buk* genes and overexpression of $adhE1^{D485G}$ gene	0.76 [a]	1.32 [g]	Jang et al. [20]
n-Butanol	*Clostridium cellulovorans*	Avicel	Overexpression of adhE2	0.39 [a]	1.42	Yang et al. [21]
Isobutanol						
	Corynebacterium glutamicum	Glucose	Overexpression of *als*, *ilvC*, *ilvD*, and *adhA*; disruption of *ldh*	NA	4.9	Smith et al. [22]
	Bacillus subtilis	Glucose	Overexpression of *G6PD*, *udhA* and *PntAB*; inactivation of *pgi*	0.37 [b]	6.12	Qi et al. [23]
	Saccharomyces cerevisiae	Glucose	Overexpression of Adh2, Aro10	15.00 [c]	0.63	Brat et al. [24]
	Saccharomyces cerevisiae	Glucose	Overexpression of *kdc*, *adh*, *Ilv2*, and *pdc1*	6.60 [c]	143 [h]	Kondo et al. [24]
	Saccharomyces cerevisiae	Glucose	Overexpression of *alsS*	NA	263.1 [h]	Park and Hahn [25]
	Escherichia coli	Glucose	Overexpression of *kivd* and *adh*.	NA	1.78	Gupta et al. [26]

Table 2. *Cont.*

Target Compound	Organism	Substrate	Genetic Engineering Strategy	Production Strategy		Reference
				Yield (g/g)	Titer (g/L)	
Isopropanol	*Escherichia coli*	Glucose	CRISPR EnAbled Trackable genome Engineering (CREATE)—upregulated *adc*, and *adh*	0.75 [d] mol/mol	7.1	Liang et al. [27]
	Corynebacterium glutamicum	Glucose	Overexpression of *thlA*, *adh*, *atoD*, and *atoA*; CRISPR-mediated edition of *ldh* *ppc*	0.34 [d]	10.25	Ko et al. [28]
Pentanol derivatives						
Isopentanol	*Corynebacterium glutamicum* ATCC 13032	Glucose	Expression of *HmgR* homolog	9.7 [e]	1.25	Sasaki et al. [29]
2-methyl-1-butanol (2-MB) and 3-methyl-1-butanol (3-MB)	*Corynebacterium glutamicum*	Glucose	Expression of *Adh* and *kdc*	0.02 [a] (2-MB) and 0.10 [a] (3-MB)	0.37 (2-MB) & 2.76 (3-MB)	Vogt et al. [30]
Diesel and jet fuels						
β-carotene	*Escherichia coli*	Glucose	CRISPR-Cas9 genome editing and integration of *crtE*, *crtB*, *crtI*, and *crtY*	NA	2.0 g/L	Li et al. [31]

[a] g/g; [b] C-mol/C-mol glucose; [c] mg/g glucose; [d] mol/mol; [e] %; [f] g//L/24 h; [g] g/L/h; [h] mg/L.

2.1. Higher Alcohols

2.1.1. Butanol

Production of alcohol by industrial microbial hosts using various renewable resources has received much attention due to its inherent potential to supply markets in response to strategic demands [32]. Owing to its high energy density, lower hygroscopicity, and volatility, butanol is considered an ideal alternative for bioethanol [33]. Furthermore, butanol is less corrosive and hence, offers safe and accessible transportation options [34]. Butanol can be produced via Clostridial ABE fermentation, which is among the oldest industrial fermentation processes. However, selective production of butanol by Clostridial fermentation remains severely impeded by lower yield, cell toxicity towards alcohols, and inhibitors from renewable feedstocks. Furthermore, the intricate genome design of these microbes makes them a challenging target for genetic engineering [35]. Thus, scientists have diverted their attention towards less complex, genetically tractable microbial hosts such as *E. coli*. Inui et al. [16] reported first the generation of butanologenic *E. coli* by expressing an array of genes involved in the ABE pathway from *Clostridium acetobutylicum* ATCC 824. The expression of *thL* (acetyl-CoA acetyltransferase), *hbd* (β-hydroxybutyryl-CoA dehydrogenase), *crt* (hydroxybutyryl-CoA dehydratase), *bcd-eftB-eftA* (butyryl-CoA dehydrogenase), and *adhe1/adhe* (butyraldehyde dehydrogenase and butanol dehydrogenase) genes under the constitutive promoter (P_{tac}) facilitated butanol production under anaerobic conditions, yielding 1.2 g/L of butanol from 40 g/L glucose, with 0.1 g/L of butyrate as a by-product. Atsumi et al. [36] reported higher butanol production by the heterologous expression of *thL*, *hbd*, *crt*, *bcd-eftB*, and *adhe2* genes from *Clostridium acetobutylicum* in *E. coli* under anaerobic conditions, resulting in 139 mg/L of butanol and improved butanol tolerance up to 1.5% of butanol in the medium.

In a study by Wen and Shen [17], the exploitation of endogenous fermentation regulatory elements (*FRE*) for 1-butanol production was illustrated in *E. coli*. These self-regulated transcription and translation regulatory elements present in the 5′ upstream region control

the expression of heterologous genes (*ldhA*, *frdABCD*, *fdh*, *adhE*, and *ackA*). Under optimal anaerobic growth decoupled conditions, the engineered strain overexpressing *fdh* (formate dehydrogenase) under *FREadhE* generated 10 g/L of 1-butanol with 0.25 g/g yield in 24 h. Shen and Liao [18] reported 1-butanol production coupled with the co-production of 1-propanol in *E. coli* by downregulating amino-acid biosynthesis using the 2-keto acid pathway and eliminating other competing pathways. They developed this strain by overexpressing *Lactococcus lactis kivd*, *S. cerevisiae adh2*, and *E. coli ilvA*, *leuABCD*, *thrA*, and *fbrBC*, followed by the elimination of homoserine O-succinyl transferase (*metA*) and threonine dehydrogenase (*tdh*). The resultant engineered strain exhibited titer up to 2 g/L of 1-butanol and 1-propanol at approximately 1:1 proportion [18].

Since butanol possesses lower volatility and higher energy density than ethanol, metabolic engineering approaches have been attempted to increase butanol production by reducing the production of other competitive by-products [33]. To achieve this, strategies involved in improving the butanol or higher alcohols production by ABE fermentation were adopted, which include the inactivation of by-products, redirection of carbon flux, and enhancement of intracellular NADP(H) level to strengthen the butanol metabolic pathway. Moreover, heterologous genes may be incorporated to convert butanol and ABE solvents into higher value-added products [37].

For example, the *Clostridium acetobutylicum* strain PJC4BK was metabolically engineered to disrupt the butyrate kinase (*buk*) gene involved in the butyrate formation pathway to improve the butanol production from 11.7 to 16.7 g/L [19]. Similarly, the overexpression of molecular chaperone *GroESL* in *Clostridium acetobutylicum* significantly enhanced solventogenesis-relevant enzyme activities and butanol tolerance, eventually improving the final butanol production by 30% and 32% compared to those of the plasmid control and wild-type strains, respectively [38]. In addition, the *Clostridium acetobutylicum* strain M5 was genetically engineered to enhance butanol production by the overexpression of *adhE1* using the promoter P_{ptb}, followed by the disruption of acetoacetate decarboxylase (*adc*), resulting in a higher ratio of butanol that was up to 82% of the total ABE solvents [39–41]. The metabolic flux of another *Clostridium acetobutylicum* strain was redirected towards the "hot channel" of the glycolytic pathway to significantly increase 1-butanol production (18.9 g/L) with 0.71 mol/mol glucose using batch fermentation [20]. Yang et al. [21] reported the first metabolic engineering of *Clostridium cellulovorans* to simultaneously produce n-butanol and ethanol directly from cellulosic biomass. This was achieved by expressing *adhE2* (alcohol/aldehyde dehydrogenase). In addition, the diversion of a metabolic shift from acid production towards alcohol production was attained by supplementing the electron carrier methyl viologen in the medium. The developed strain produced 1.42 g/L of n-butanol and 1.60 g/L ethanol in a consolidated bioprocessing approach.

2.1.2. Isobutanol

Isobutanol possesses high energy density, lower hygroscopy, and relatively lower toxicity than ethanol, which supports its potential application as a hydrocarbon fuel [42]. The degradation of amino acids in the Ehrlich pathway for the production of keto-isovalerate is the primary metabolic pathway for isobutanol production [43]. 2-ketoisovalerate, the 2-keto-acid precursor required for valine biosynthesis, is also generated with isobutanol production in *Corynebacterium glutamicum* [30]. Smith et al. [22] attempted to engineer *C. glutamicum* to convert 2-ketoisovalerate into isobutanol, and also analyzed the strain's tolerance against isobutanol titer. The overexpression of ketoacid synthesis pathway-relevant genes, including acetolactate synthase (*als* from *B. subtilis*), ketol-acid reductoisomerase (*ilvC*) and dihydroxy-acid dehydratase (*ilvD*) from *C. glutamicum*, and alcohol dehydrogenase (*adhA* from *Lactococcus lacti*) resulted in 2.6 g/L of isobutanol production. In addition, in the resultant *C. glutamicum* strain, the intracellular lactate dehydrogenase (*ldh*) gene was disrupted to redirect the carbon flow from lactate, which improved isobutanol titer (4.9 g/L) up to ∼25%. Qi et al. [23] engineered the *B. subtilis* strain to enhance isobutanol production up to 6.12 g/L in fed-batch fermentation by overexpressing the glucose-6-

phosphate dehydrogenase gene (*G6PD*) and transhydrogenase genes (*udhA* and *PntAB*), along with the inactivation of glucose-6-phosphate isomerase gene (*pgi*). These genetically engineered *B. subtilis* strains could convert amino acids into isobutanol via secretory protease-mediated polypeptide hydrolysis. Moreover, the resultant strain generated higher biofuels (2-methylbutanol and 3-methylbutanol) along with fertilizer ammonia.

Besides its well-established role as an ethanol producer, *S. cerevisiae* has been engineered to generate higher alcohols. For instance, Brat et al. [24] obtained isobutanol titer of 0.63 g/L by relocating valine biosynthetic enzymes (*Ilv2*, *Ilv5*, and *Ilv3*) from mitochondria to cytosol with the overexpression of essential genes for isobutanol production [alcohol dehydrogenase (*Adh2*) and ketoisovalerate decarboxylase (*Aro10*)].

Elevated isobutanol production can also be obtained by enhancing the endogenous Ehrlich pathway to alter the ethanol carbon flux via pyruvate [44]. A 13-fold increment of isobutanol production with a yield of 6.6 mg/g was achieved by overexpressing keto acid decarboxylase (*kdc*), alcohol dehydrogenase (*adh*), and acetolactate synthase (*Ilv2*), together with the deletion of pyruvate decarboxylase (*pdc1*) within *S. cerevisiae*. In another study by Milne et al. [45], isobutanol production was improved by overexpressing a series of decarboxylase-encoding genes [2-oxo-acid-decarboxylase (*ARO10* from *S. cerevisiae*), α-ketoisovalerate decarboxylase (*kivD*), and *KdcA* from *L. lactis*] engaged in ketoisovalerate decarboxylation. Among these, the *KdcA* gene was determined to be a potential decarboxylase to actively engage in the production of isobutanol and other branched/linear chain alcohols [45]. Park and Hahn [25] enhanced isobutanol yield in *S. cerevisiae* by integrating a synthetic cytosolic isobutanol pathway comprising *alsS* from *B. subtilis* as well as native *ilv3* and *ilv5* genes. Overexpression of *alsS* resulted in α-acetoacetate accumulation, which severely impeded cell growth. The controlled expression of *alsS* under copper-inducible CUP1 promoter was found to circumvent this issue. Under optimized promoter induction, the engineered *S.cerevisiae* strain was capable of producing 263.2 mg/L of isobutanol, which was 3.3-fold higher than the control.

Besides Baker's yeast, attempts have been made to genetically modify *Zymomonas mobilis* for isobutanol production using *kivD* and *adhA* genes from *L. lactis* [46]. Qiu et al. [47] studied the overexpression of native *ilvC* and *ilvD*, along with heterologous *alsS* expression in *Z. mobilis* expressing the *kdcA* gene. The generated artificial operon kept under the constitutive expression of the P_{gap} promoter significantly redirected the carbon flux from ethanol production towards isobutanol production with a final titer of 4.0 g/L. More recently, a consolidated bioprocessing approach for isobutanol production in *E. coli* has been reported using CRISPR technology. The integration of the α-KIV pathway was performed using CRISPR-Cas9 technique, followed by the downregulation of the competitive valine biosynthetic pathway through CRISPR technology. This simultaneous dual genetic approach aided in the generation of 1.78 g/L of isobutanol with 93% yield and productivity of 0.07 g/L/h [26].

2.1.3. Isopropanol

Isopropanol is another commercial alcohol that can be used as an ancillary fuel. It is also regarded as a potential candidate to replace methanol in oil transesterification to produce biodiesel [48]. It does not crystallize at low temperatures and is currently refined from petroleum, causing dependency on an already declining resource [49]. Owing to its high energy density (33.4 MJ kg^{-1}), isopropanol attributes its potential as a drop-in fuel in internal combustion engines [50]. Some strains, such as *Clostridium beijerinckii*, can produce isopropanol during the isopropanol–butanol–ethanol (IBE) fermentation process; however, the production titer is low, i.e., approximately 2.4 g/L [51]. Hence, enthusiasm has increased to produce isopropanol in other microorganisms, particularly in *E. coli* and yeast. CRISPR-enabled tractable genome engineering (CREATE) was reported in *E. coli* for isopropanol production by Liang et al. [27]. CREATE can be considered to be derived from multiplex automated genomic engineering (MAGE) reported by Wang et al. [52]. MAGE could introduce several simultaneous gene disruptions at many locations in the

chromosomes of target strains, resulting in the generation of billions of combinatorial variants in a few days, and the incorporation of CRISPR-Cas9 and barcoding with MAGE could facilitate the CREATE process, which could generate hundreds of thousands of designer variants with phenotype- and barcode-mediated selection in a shorter time at low cost [53]. Liang et al. [27] reconstructed the isopropanol synthetic pathway via CRISPR in *E. coli* by synthesizing approximately 1000 designer variants containing combinatorial ribosome binding site mutants of five different genes (*thl*, *atoDA*, *ctfAB*, *adc*, and *adh*) derived from *E. coli* and *Clostridium* sp., and obtained the best variant PA14, with predominantly upregulated *adc* and *adh* genes, reaching maximum isopropanol productivity of 0.62 g/L/h.

In addition to the above developments, the CRISPR-Cas toolkit has extended its specificity towards multiple gene deletions and integration of large gene fragments in industrial microbial hosts, with improved carbon flux toward alcohol production. Recently, Ko et al. [28] demonstrated the reallocation of carbon flux from the central metabolism for the enhanced production of isopropanol in *Cornyebacterium glutamicum* through CRISPR-based approaches. Firstly, isopropanol biosynthesis was achieved by heterologous overexpression of the acetate-dependent pathway genes acetyl-CoA acetyltransferase and acetoacetate decarboxylase (*thlA* and *adh* from *Clostridium acetobutylicum*), α- and β-subunits of acetate CoA-transferase (*atoD* and *atoA* from *E. coli*), and NADP(H)-dependent secondary alcohol dehydrogenase (*sdh* from *Clostridium beijerinckii*). Additionally, biosynthetic gene expression was improved by replacing the P_{tac} promoter with a high-strength P_{H36} promoter, which allowed the engineered strain to direct more carbon flux toward isopropanol production. However, the resultant strain had enhanced by-products (succinate and lactate) yield, which could be overcome by CRISPR-Cas12a-aided genome editing of *ldh* (lactate dehydrogenase) and *ppc* (phosphoenolpyruvate carboxylase). In this study, dual-stage fed-batch fermentation coupled with gas stripping attained 97.68% isopropanol production with 0.34 mol/mol yield and 0.1 g/L/h productivity.

2.2. Pentanol Derivatives

Pentanol derivatives with higher octane number, such as 2-methyl-1-butanol (2–MB), 3-methyl-1-butanol (3–MB), and isopentenol, from engineered *Corynebacterium glutamicum* strains with enhanced tolerance against the toxicity of generated products are also advanced biofuels. Vogt et al. [30] engineered *Corynebacterium glutamicum* strains to over-produce 2–MB and 3–MB from their respective 2-keto acids through the combined expression of alcohol dehydrogenase (*adh*) and keto acid decarboxylase (*kdc*) genes. Under oxygen distress, the resultant *Corynebacterium glutamicum* strain generated 0.37 g/L and 2.76 g/L of 2–MB and 3–MB, respectively. Similarly, by determining the correlation between the enzyme 3-hydroxy-3-methylglutaryl-coenzyme–A reductase (*HmgR*) and isopentenol titer, Sasaki et al. [29] enhanced isopentenol production to 1.25 g/L in *Corynebacterium glutamicum* ATCC 13032 by substituting an NADH-dependent *HmgR* homolog from *Silicibacter pomeroyi* with further development of Δ*poxB* Δ*ldhA* host.

2.3. Diesel and Jet Fuel

Isoprenoids (C_5H_8), also known as terpenoids, comprise thousands of different compounds with a broad scope of industrial applications. The metabolic pathway of isoprenoids produces a broad spectrum of products ranging from branched-chain alkanes and cyclic alkanes (sesquiterpenes), alkenes (monoterpenes, diterpenes), and alcohols (farnesol, geraniol, isopentenol). Owing to their higher energy content, low hygroscopic properties, and exceptional fluidity at low temperatures, isoprenoid compounds can be developed as additives for gasoline over ethanol and have the potential to replace diesel or jet fuel in the future [54]. Morais et al. [55] compared the greenness of isoprenoid production between the existing petrochemicals and a new biological process mediated through a modified *E. coli* strain. They found the latter process to be more favorable in terms of material and energy efficiency, although the calculated cost was slightly higher than the market price due to the utilization of waste biomass.

Isoprenoids are ubiquitously produced by humans, plants, yeasts, and bacteria [56]. The isoprenoid backbone in prokaryotes is usually synthesized through the deoxyxylulose 5-phosphate (DXP) pathway, whereas most eukaryotes use the mevalonate (MVA) pathway [57]. However, the fundamental building blocks for isoprenoid production are isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), irrespective of the choice of organism. In the past decades, researchers have constantly been involved in the construction of isoprenoid-producing microorganisms by overexpressing the non-native isoprene synthase or engineering the isoprenoid precursor pathway to improve the titer for the isoprenoid-based biofuels [58]. To complement these, targeted genome engineering approaches such as CRISPR-Cas9-based genome engineering have been used for production and purity enhancement.

MVA is a key intermediate involved in converting acetyl-CoA to isopentenyl 5-diphosphate, a precursor in the production of isoprenoids in the MVA pathway [59]. Jakočiūnas et al. [60] first obtained an immense 41-fold increase in MVA production from *S. cerevisiae* via the CRISPR technology in various combinations of gene disruptions in five different genomic loci. It was accomplished without the overexpression of any gene involved in MVA production, thus demonstrating an exploratory step into CRISPR-based multiplex genome engineering in isoprenoid production. Li et al. [31] accomplished CRISPR-Cas9-based genome editing for isoprenoid production with 100% efficiency by integrating the β-carotene synthetic pathway into *E. coli*. The process involved the introduction of four genes (*crtE*, *crtB*, *crtI*, and *crtY*) from *Pantoea agglomerans* into *E. coli* [61], followed by the improvement of methylerythritol-phosphate (MEP) pathway flux as well as the modification of the glucose transport system, resulting in 33 genomic changes and over 100 mutant strains. The best producer, comprising 15 targeted improvements, produced 2 g/L of β-carotene in fed-batch fermentation, which was 8.4-fold higher than the control strain. Almost at the same time, a proof of concept study was carried out by Ronda et al. [62], in which the CRISPR/Cas9-based genome editing methodology was adapted to integrate a non-native β-carotene synthetic pathway for isoprenoid production in *S. cerevisiae*. Herein, three genes, viz., *BTS1* (native GGPP synthase), *crtYB* (phytoene synthase/lycopene cyclase), and *crt1* (phytoene desaturase) were engineered simultaneously to achieve 84% of positive transformants. They also improved the locus specificity and efficiency of CRISPR gene integration by up to 100%. These results showcase the bright prospects of utilizing CRISPR-Cas9-based genome engineering to regulate cellular metabolism for improved isoprenoid production. However, toxicity and metabolic burden are significant problems to be considered during the overproduction of isoprenoids in the hosts with multiple tolerance mechanisms [62]. Therefore, further investigation should be focused on combating toxicity and improving isoprenoid tolerance without compromising productivity.

3. Metabolic Pathway Engineering Strategies for Advanced Biofuels Production

Among the various types of biomass used as substrates, primary LCB, along with agro-industrial wastes, have attracted considerable attention due to its abundance and sustainability. The principal components of LCB are cellulose (a glucose polymer) and hemicellulose (a mixture of hexoses and pentoses), which are tightly linked by the heterogeneous polymer of phenylpropanoid lignin subunits [63]. The presence of lignin effectively hinders the microorganism from utilizing LCB, requiring an intermediary step of enzymatic or chemical hydrolysis. The resultant hydrolysate consists of monomeric sugars that can be used as feedstock for microbial fermentation. However, the significant bottlenecks which hinder the industrial application of LCB were the inhibitors produced during the chemical hydrolysis and cost-inefficient enzymatic treatment. Therefore, engineering microorganisms with inherent metabolic pathways for simultaneous saccharification and fermentation were carried out to improve the economic prospects of the process. Table 3 summarizes the metabolic engineering strategies employed to advance biofuel production.

Table 3. Key metabolic engineering strategies applied to enhance advanced biofuel production.

Organism	Strategy	Achieved Target	Reference
Modifying sugar preference through transporter manipulation			
Saccharomyces cerevisiae	Production of 2, 3-Butanediol from cellobiose	Integrating cellobiose and 2,3-butanediol (2,3-BDO) pathways in a pdc-deleted mutant enables efficient utilization of cellulosic sugars, resulting in 2,3-BDO production, excluding ethanol production.	Nan et al. [64]
Saccharomyces cerevisiae strain BSW2AP	Improved pentose utilization in *S. cerevisiae*	The *lat-1* and *Mtlat-1* genes, encoding dual L-arabinose transporters, enhance pentose utilization and ethanol production from lignocellulosic hydrolysates.	Li et al. [65]
Saccharomyces cerevisiae	Enhance the D-galacturonic acid (d-GalUA) consumption for valorization of pectin-rich agro-industrial residues.	Expression of heterologous pathway for d-GalUA transporter protein in NAD-dependent glycerol pathway maximized consumption rate.	Perpelea et al. [66]
E. coli	Efficient lignocellulosic fermentation (both primary and secondary sugars) by *E. coli* biocatalysts	XylR mutation in E. coli enhanced xylose uses 4-fold, independent of carbon catabolite repression (CCR).	Sievert et al. [67]
Engineering microbes for improved tolerance			
E. coli	Using acetate as the sole carbon source	ALE adapted *E. coli* to utilize acetate as the sole carbon source, enhancing growth and altering RNA polymerase interaction.	Rajaraman et al. [68]
Kluyveromy-ces marxianus JKH5	Development of multiple inhibitor tolerant yeast via ALE for sustainable bioethanol production	ALE-adapted strain ferments unwashed biomass for sustainable bioethanol, with acetic acid, furfural, and vanillin tolerance.	Patel et al. [69]
E. coli	Generating IL-tolerant microbial hosts simplifies downstream carbon conversion for target compounds.	The ALE-evolved strain found *cydC* gene mutation, enabling IL-derived sugar tolerance.	Eng et al. [70]
Saccharomyces cerevisiae	Enhance tolerance to higher alcohols	Evolved *S. cerevisiae* to tolerate n-hexanol; mutations in translation initiation proteins improve tolerance to medium-chain alcohols.	López et al. [71]

3.1. Modification of Sugar Preference via Transporter Engineering in Microbes

Besides the glucose-rich cellulose component, the hemicellulose component of biomass, which accounts for 45%, contains a mixture predominantly consisting of pentoses, such as xylose and arabinose, followed by hexose sugars [72]. Even though microbial species utilize pentose sugars for biofuel production [73], the catabolism of these sugars is typically suppressed by the preferential utilization of hexoses over pentoses, owing to allosteric competition or carbon catabolite repression (CCR) in sugar transport [74]. Thus, the selective or sequential utilization of sugar during the fermentation process results in the underutilization or accumulation of non-preferred sugars, which often reduces the fermentation product yield [75,76]. Thus, the correlation between sugar input and product output is critical for improving consolidated bioprocessing, along with extracellular biomass utilization and metabolic pathways. Therefore, sugar transporters are crucial factors determining the variety of sugars and their selectivity, which facilitates efficient input and output activities. Hence, sugar transporter engineering could allow the simultaneous utilization of various sugars present in biomass hydrolysate, improving the overall saccharification and fermentation of biofuel production.

In general, the transporter proteins are crucially involved in selective permeability of nutrients and metabolites, including sugars, by both passive and active transport across the cell membrane [77]. Biomass hydrolysate obtained after pretreatment often comprises mixed sugars; the selectivity of sugars for biofuel fermentation is usually determined by the sugar transporter present in the microorganism. In industrial biofuel production, the range of substrates utilized for fermentation is often determined by the types of transporters present in the microbes. Yeast, a well-known workhorse of industrial fermentation and an excellent ethanol producer, has been extensively engineered to convert biomass directly into value-added products. Genetic engineering of sugar transporters for the selective utilization of sugars enhances biomass-mediated bioprocessing for biofuel production. Thus, Nan et al. [64] exhibited the expression of β-glucosidase and cellodextrin transporters from *Neurospora crassa* for the selective conversion and uptake of cellobiose by *S. cerevisiae*.

A consolidated bioprocessing approach has been applied to utilize L-arabinose by expressing dual L-arabinose transporters genes, viz., *lat-1* from *N. crassa*) and *Mtlat-1* from *Myceliopthera thermophila*, alongside proton symporter in *S. cerevisiae*. Specifically, the resulting strain *MtLAT-1* appeared to express higher specificity towards L-arabinose, reduced inhibition by D-glucose, and generated higher ethanol than the control [65]. Sugar-rich pectin wastes, viz., citrus and sugar beet pulps produced from the food industries, contain a large proportion of D-galacturonic acid (d-GalUA) formed via galactose oxidation [78]. Biorefinery approaches allow these pectin wastes to efficiently serve as a source of alcohol production due to the low lignin and high sugar monomer ratio. However, the alcohol-producing *S. cerevisiae* is unable to transport d-GalUA. Hence, several strategies have been made to engineer d-GalUA transporters and their corresponding pathways in *S. cerevisiae* for ethanol production. Biz et al. [79] reported recombinant *S. cerevisiae* expressing a d-GalUA transporter protein, *gat1*, from *N. crassa* along with d-GalUA catabolism genes from *Aspergillus niger* (*gaaA*, *gaaC*, and *gaad*) and *Trichoderma reesei* (*lgd1*) for ethanol production using d-GalUA. However, these heterologous transporter expressions did not significantly improve the d-GalUA consumption and ethanol production in *S. cerevisiae*. The conversion of d-GalUA into distinctly reduced alcohols requires a considerable level of electrons for the generation of NAD(P)H as intracellular reducing equivalents [80]. This issue has been addressed by engineering *S. cerevisiae* containing the NAD-dependent glycerol pathway by expressing d-GalUA transporters and their corresponding enzymes. The resultant strain readily consumes both d-GalUA (at the rate of 0.23 g $gCDW^{-1}$ h^{-1}) and glycerol (source of electrons) with a theoretical yield of 70% ethanol [66].

The utilization of cellobiose in *S. cerevisiae* GH1-1 is achieved by expressing mutant cellodextrin transporters (*Cdt-1* or *F213L*) via an evolutionary engineering approach, which facilitated enhanced uptake of cellobiose over the parental CDT-1 strain. The engineered strain demonstrated efficient simultaneous saccharification and fermentation, producing 37.3 g/L ethanol using cellobiose [81].

Despite the advancements in yeast sugar transporters, bacterial sugar transporter engineering for biofuel production is far less reported. Among the various transporters, the bacterial-specific phosphoenolpyruvate: carbohydrate phosphotransferase (*PEP–PTS*) system is predominantly involved in the phosphorylation and transportation of hexoses and their derivatives [82]. However, few bacteria that possess the capabilities to utilize pentoses, such as arabinose and xylose, use distinct transporters other than PTS [83]. The expression of ABC transporters in *Clostridium thermocellum* is specifically involved in the movement of pentose oligosaccharides [84]. In *E. coli*, independent ABC transporters for xylose (*XylFGH*) [85] and arabinose (*AraFGH*) [86] are present, along with their proton-associated symporters (*XylE* and *AraE*) [87]. Henderson [88] reports that proton-associated galactose transporter *GalP* catalyzes and transports xylose in *E. coli*. Another strategy to improve mixed sugar utilization by bacteria is to alleviate its dependency on CCR [89]. This technique allows the microbes to co-utilize pentoses even in the presence of glucose, significantly improving their preferences for LCB-derived sugars. Sievert et al. [67] demonstrated this concept by introducing a point mutation in the transcriptional activator for

xylose catabolic operon (*XylR*) in *E. coli*. The resultant strain displayed its independence towards CCR and enhanced xylose utilization by 4-fold in the glucose–xylose mixture than the control. This study substantiates the ability of transformant *E. coli* to utilize sugar mixtures derived from cost-efficient renewable feedstocks for fermentation.

The previous reports primarily focused on utilizing model or pure cellulose compounds, which require minimal enzymes for substrate hydrolysis in biofuel generation. In a study by Lee et al. [90], a cellulose-adherent Saccharomyces cerevisiae strain was engineered to display four synergistic cellulases (BGL, EG, CBH1, and CBH2) on its cell surface through cell-surface display technology. This modified yeast strain exhibited robust adhesion to cellulose, leading to enhanced hydrolysis efficiency. By capitalizing on this strong cellulose adhesion, a consolidated bioprocessing approach was developed for ethanol production from rice straw, significantly reducing enzyme dosage (40%) during fermentation. However, proximity limits the efficiency of synergistic multi-enzyme assemblies through the cell-surface display. Therefore, determining the optimal inter-enzyme distance is crucial for maximizing enzyme density and enhancing cellulose hydrolysis. To address this, Smith et al. [91] developed a novel quantitative approach to characterize whole-cell biocatalysts and investigate the formation of yeast-surface displayed multi-enzyme assemblies. The study revealed that proximity effects are synergistic only when the average inter-enzyme distance is >~1130 nm. These findings pave the way for advancing biocatalyst engineering by transitioning from a trial-and-error approach to a more rational design. These findings present a promising strategy for enhancing cellulose hydrolysis and advancing the feasibility of cellulosic biofuel production.

3.2. Engineering Microbes for Enhanced Tolerance towards Substrates and Products

The generation of inhibitors at various stages of processes, viz., substrate pretreatment, end-product accumulation, and by-product generation, severely impedes the production of advanced biofuels at the industrial scale [92]. Further, the expression of a heterologous pathway for advanced biofuels in non-native hosts creates an imbalance in intracellular energy intermediates and redox potential, constantly increasing the metabolic burden [56]. Hence, advancing microbial tolerance toward these inhibitions is highly imperative for the enhanced productivity of advanced biofuels. Various strategies have been implemented to achieve microbial tolerance towards inhibitors, such as adaptive laboratory evolution (ALE), genome shuffling, and random barcode transposon-site sequencing [93]. ALE is one of the most powerful tools for improving microbial phenotypes based on evolutionary selection without prior knowledge about the organisms [94]. Implementation of ALE to develop enhanced tolerance towards inhibitors generated during the biomass pretreatment was illustrated by Rajaraman et al. [68]. In this study, a pseudo-steady-state ALE has been deployed for adapting an *E. coli* to utilize acetate as the sole carbon source. The evolved strain *E. coli* MEC136 demonstrated a faster growth rate (~25%) over the parent strain in a medium supplemented with 85 mM acetate. Genome sequencing analysis displayed a single amino acid modification in RpoA in the α subunit of the RNA polymerase. The identified mutation altered serine to proline in RpoA and modified the interaction of RNA polymerase enzymes with global transcriptional activators, enabling transcriptional changes to utilize acetate.

Furthermore, the microbial strains that tolerate these inhibitors also act as potential candidates for multiple inhibitor tolerance for sustainable fuel production. Recently, Patel et al. [69] demonstrated ALE's ability to generate *Kluyveromyces marxianus* JKH5 that tolerates numerous inhibitor compounds to produce biofuels. The ALE-adapted *K. marxianus* JKH5 C60 strain sustained acetic acid, furfural, and vanillin supplemented in the cocktail medium at concentrations of 3, 1, and 1 g/L, respectively. The resultant strain displayed a reduced lag phase and a 3.3-fold higher specific growth rate than JKH5. When the developed strain was provided with unwashed dilute acid–alkali pretreated sugarcane bagasse in the presence of inhibitors, it produced 54.8 g/L ethanol with a yield of 0.4 g/g.

Hence, ALE can efficiently generate microorganisms that generate sustainable fuels from various renewable biomass, even in the presence of inhibitors.

Besides known inhibitors, the catalysts utilized for pretreatment also tend to inhibit the enzymatic machinery and microbial fermentation [95]. Ionic liquid (IL)-mediated biomass pretreatment is considered an efficient and green approach for the selective removal of lignin and promotes cellulose fractions for further fermentation [96]. However, the remaining ILs in sugar fractions severely impede the fermentation process [97]. Eng et al. [70] addressed this issue by conferring IL tolerance to *E. coli* using ALE, resulting in the production of higher fuels, viz., D-limonene and isopentenol. Whole genomic analysis revealed that a single mutation in the cytochrome assembly factor cydC, a major subunit of the ABC transporter complex, confers the tolerance towards IL. In the presence of IL, 1-ethyl-3-methyl-imidazolium acetate-pretreated biomass, the ALE-adapted *E. coli* with the *cydC-D86G* mutation produced D-limonene and isopentenol at the concentrations of 200 mg/L and 350 mg/L, respectively.

Another major challenge associated with higher biofuel production is the toxicity of generated biofuels (end-product) toward the fermentation host [98]. Hence, alleviating end-product toxicity in a host is important to attain sustainable production. López et al. [71] attempted to alleviate the medium-chain alcohol tolerance in *S. cerevisiae* BY4741 by ALE. The strain BY4741 evolved by ALE showed enhanced n-hexanol tolerance (0.15–0.2%) compared to the parental strain, and also rendered tolerance against various ranges of advanced medium-chain biofuels (propanol to octanol). Reverse genome engineering revealed that the mutation occurring in the translation initiation protein genes of *eIF2B* [*Gcd1p* (γ subunit), *Gcd7p* (β subunit)] and *eIF2* [*Sui2p*, α subunit] are involved in alleviating the tolerance. Hence, ALE has been proved to efficiently generate microorganisms that produce advanced sustainable fuels from various renewable biomass.

Thus, developing new strains capable of tolerating higher inhibitor or substrate concentration along with the feed-back inhibition of biofuels will significantly improve the economic feasibility of the bioprocess. Further, the implication of genome engineering techniques and genome sequence information will widen our knowledge of the host microbe, which facilitates an untapped biomass spectrum with improved biomass saccharification (Figure 2). Research and development of several novel sugar transporters along with modified saccharifying enzymes with improved potential can enhance the applicability of consolidated bioprocessing for biofuel production.

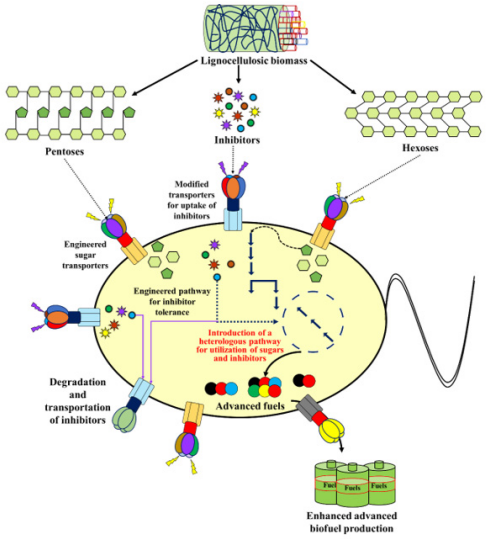

Figure 2. Genetic engineering strategies implemented for improved sugar transportation and inhibitor tolerance for advanced biofuel production. Expression of heterologous pathways for the utilization of pentose sugars and the metabolic pathway engineering for inhibitors tolerance for the enhanced production of advanced biofuels from the renewable lignocellulosic biomass.

4. Chemo-Catalytic Conversion of Primary Biofuels into Advanced Biofuels

Compared to fossil reserves, renewable LCB feedstocks often have higher oxygen level, and relatively lower energy density, which hinders their direct conversion into biofuels [99]. Converting complex LCBs via thermochemical and biological pretreatment generates a mixture of sugars used to produce fuels and other value-added products. Microbial conversion of LCB-derived sugars into primary alcohols (ethanol and butanol) has been improved via genetic manipulation strategies to attain the near-theoretical maximal yield [34]. However, the energy density and volatility properties of short-chain alcohols are not aligned well with those of the advanced fuels used in the aviation sector [100]. Hence, various metabolic engineering techniques are adopted to produce advanced long-chain biofuels, which often suffer from intolerance towards substrates and inhibitors, low titer productivity, and yield [8]. Indeed, ABE generated via microbial fermentation can be converted into long-chain alkanes through the alkylation of nucleophilic α-carbons in acetone with the electrophilic α-carbons in butanol and ethanol (Figure 3). Hence, integrating ABE fermentation with chemical catalysis also includes efficient downstream processes to attain sustainable advanced biofuel production [101].

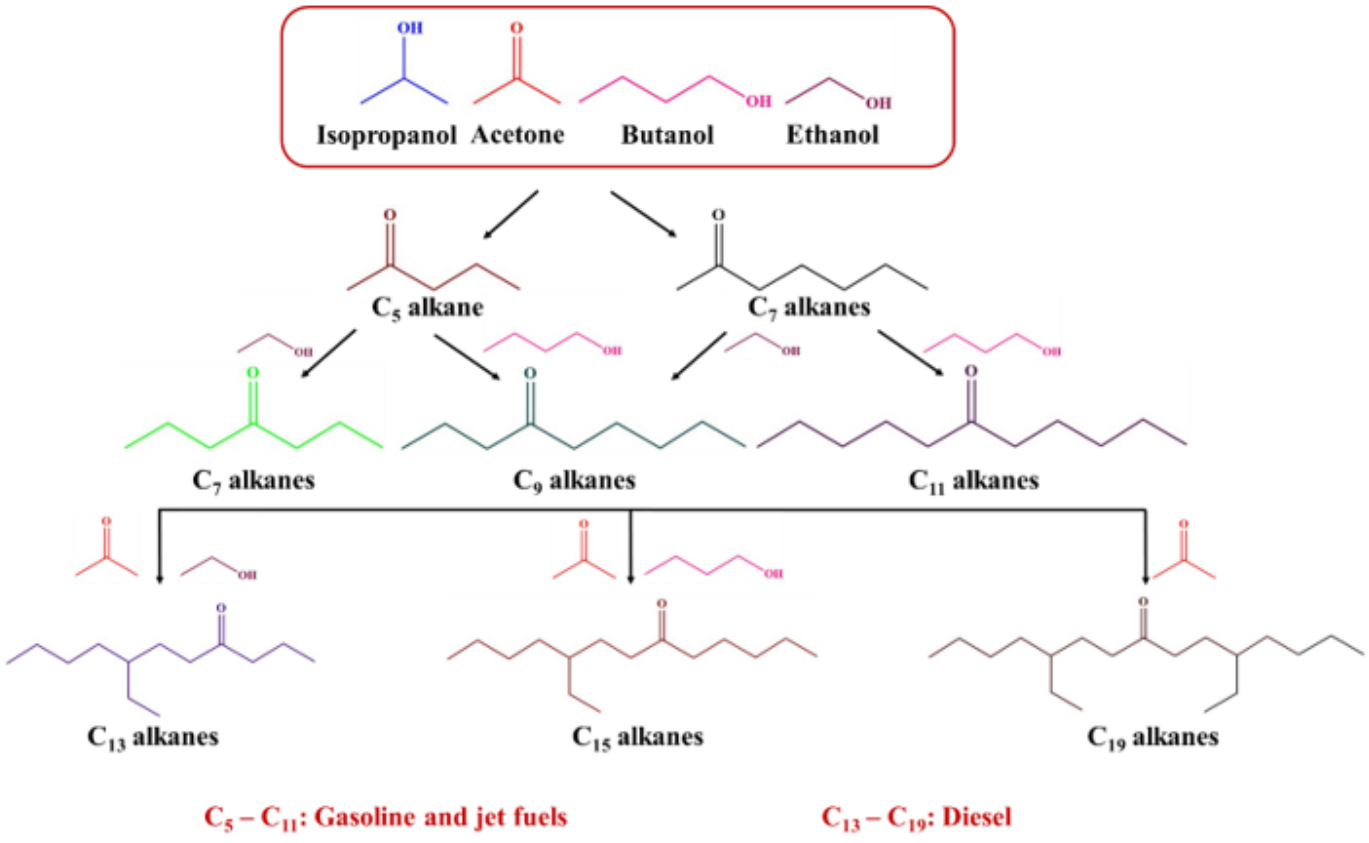

Figure 3. General reaction scheme for the chemocatalytic upgradation of ABE products into higher biofuel production. Dehydrogenation and aldol condensation of ABE/IBE by metallic catalyst generates gasoline and jet fuels (C_5–C_{12}) and diesel range (C_{13}–C_{19}) fuels.

4.1. Pd and Cu-Catalyzed Alkylation

Sreekumar and co-workers reported a combination of hydrotalcite (HT)-assisted Cu (II) and Pd (0) catalysts to generate an enhanced concentration of diesel components through the alkylation of modified fermentation products IBE [102]. The combinatorial activities of chemical catalysis with modified IBE induced the improved selectivity of C_{12+} diesel components. The alkylation of IBE by heterogeneous recyclable copper–hydrotalcite (Cu–HT) catalyst, specifically involved in the in situ formation of acetone from isopropanol dehydrogenation. The passive formation of acetone eventually improved butanol dimerization

to 2-ethyl hexanol, which increased the selectivity towards C_{12+} components. The results show that the concentration of C_{12+} ingredients in the IBE product mixture was 25 wt%, which was a 2-fold improvement over the ABE mixture. Furthermore, the application of oleyl alcohol also elevated IBE components from the fermentation mixture.

The same group also developed a Pd–HT catalyst to transform a concentrated ABE mixture from *Clostridium acetobutylicum* into an advanced biofuel precursor. They utilized a high-pressure Q-type reactor for the alkylation of acetone with butanol and ethanol with a Pd–HT catalyst. Extracts from fed-batch reactors were upgraded to higher ketones, which were simultaneously distilled to obtain 86% yield. Liu et al. [103] demonstrated the alkylation efficiency of nitrogen-doped carbon-assisted Pd (PdC_{l2}/S_1-200-H_2) catalyst for the upgradation of IBE into higher ketones/alcohols. The synthesized PdC_{l2}/S_1-200-H_2 catalyst was exceptional in upgrading short-chain alcohols into higher alcohols (C_8–C_{19}) with 90% selectivity. Synthesis of C_8–C_{19} products is attained via various reactions, viz., mono-, double-alkylation, self-condensation, and trimolecular condensation of IBE for the generation of 2-heptanone/heptanol (C_7), 2-ethylhexanal/2-ethylhexanol, 6-undecanone/undecanol (C_{11}), and $>C_{12}$ components, respectively. The proposed methodology exhibits its applicability for the production of biomass-derived long-chain ketones/alcohols by the upgradation of short-chain alcohol mixtures.

Goulas et al. [104] demonstrated the ability of base-supported metal catalysts involved in aldol condensation of ABE for the generation of higher ketones as precursors for drop-in diesel. By increasing the surface area of HT-supported Cu catalysts, the reaction rates were significantly improved. The increase of Cu doping up to 2.5% significantly improved the ketone yield and the selectivity towards 2-ethyl hexanol and 4-ethyl-2-nonanone. However, increasing Cu concentration beyond the optimal level (2.5%) did not significantly improve the ketone yield and reduced 2-ethyl hexanol selectivity. This undesirable impact is possibly due to hemiacetal formation over HT's primary sites, followed by Cu-catalyzed dehydrogenation. This result indicates that the dehydrogenation rate-determining step is the cleavage of the CH bond. Butyraldehyde aldol condensation with acetone occurs by a balanced enolate formation over the HT surface, followed by a rate-limiting abstraction of the surface protons to form ketols. Afterward, the ketol is easily dehydrated to form unsaturated ketone.

4.2. Pt/Nb-Catalyzed Alkylation

Besides using LCB-derived ABE mixture to generate advanced fuels, Sreekumar et al. [102] demonstrated a new route for drop-in diesel/jet fuel production using alcohols and furfurals. The furfurals generated from the degradation of pentose sugars are combined with alcohol dehydrogenation–aldol condensation to produce furans using recyclable transition metal-free HT catalysts. As synthesized, furan derivatives were hydro-deoxygenated over the heterogeneous catalyst platinum on niobium phosphate ($Pt/NbOPO_4$) to produce higher hydrocarbons. Furanyl aldehyde hydro-deoxygenation under optimal conditions obtained the combination of cyclic and acyclic alkanes with 75–78% yield of C_8–C_{19} compounds. This study demonstrates the efficiency of utilizing alcohols and inhibitors derived from LCB to produce advanced biofuels.

Furthermore, the authors used life cycle assessment simulating a Brazilian sugarcane biorefinery to understand this approach. Based on their results, the process could curtail 53–79% of GHG emissions compared to traditional petroleum fuels, in addition to contributing a renewable source of eco-friendly diesel/jet fuel.

4.3. Fe, Co, Ni, Cu, and Zn-Catalyzed Alkylation

The generation of energy-intensive higher fuel precursors from ABE mixtures via aldol condensation is an established approach [105]. The nucleophilic α–carbons and electrophilic β–carbon of acetone and alcohols present in the ABE mixture are involved in the aldolization reaction. Typically, aldol condensation occurs in two steps, viz., dehydrogenation of alcohols into aldehydes over metallic surfaces, followed by the condensation

of aldehydes with ketone [106]. Among the reported metals (Ru, Pd, Fe, Co, Ni, Cu, and Zn) for alcohol dehydrogenation, Cu and Pd have higher catalytic activity. However, these metals are often restrained in large-scale production due to poor selectivity towards products and the generation of side products, which creates difficulties in downstream processing [107]. Further, the availability and cost of the metals used in the synthesis of catalysts play a major role. Hence, a low-cost, efficient, recyclable catalyst with high selectivity is of prime necessity. Developing sustainable catalysts with enhanced water tolerance also needs to be considered while commercializing the process. Zhu et al. [108] attempted to improve the water tolerance of a metal-loaded catalyst used to convert a solvent-free ABE mixture into higher alcohols and aimed to replace expensive Pd with abundantly available transition metals (Fe, Co, Fe, Cu, Zn, and Ni). Specifically, they utilized MgO–SiO_2, known for its modulating acid-base properties, feasibility, and stability in its application for the butadiene production at industrial scale from ethanol via aldol condensation [109]. Among the screened metals, 10% Ni-loaded Ni–MgO–SiO_2 catalyst showed up to 3% improved water resistance and 81.8% ABE conversion to yield 72.7% C_5–C_{15} alcohols and ketones. The recycling studies revealed that the Ni–MgO–SiO_2 catalyst retained its activity (without any loss) for three cycles. The higher stability can be correlated to the formation of magnesium silicates from the interaction between Ni, SiO_2, and MgO. The efficiency and selectivity of tin-doped ceria (Sn–ceria) for the conversion of ABE into 4-heptanone (4-HPO) was investigated in the presence of water by Wang et al. [110]. In simulations, ABE with water (9:51:1:22) could effectively convert 70% of the carbon from ABE into 4-HPO with 86% selectivity. In a continuous reaction (300 h), the Sn–ceria catalyst could maintain 50% carbon conversion with 86% of selectivity for 4-HPO by >90% carbon balance. Liu et al. [111] developed a bi-metal- (Co–Ni) strategy assisted by an Mg–Al oxide catalyst to convert tailored ABE into energy-intensive fuels. The increased pore diameter in the synthesized catalysts greatly influenced the electron transfer through Co–Ni, which enhanced the dehydrogenation activity. Under optimal reaction conditions, Co–Ni supported by Mg–Al oxide (MgO–Al_2O_3) catalyst could selectively convert ABE fermentation products into C_5–C_{11} alcohols and ketones via aldol condensation with a total selectivity of 90%. Furthermore, the stability of the catalyst was enhanced by simple hydrogen reduction [111]. Recently, Wu et al. [112] investigated the potential of heterogeneous MgO–Al_2O_3 mixed metal catalyst with Ni nanoparticles for upgrading ABE mixtures into long-chain (C_5–C_{15}) ketones. The synthesized catalyst with the molar ratio of 3 for Mg/Al and 6 wt.% loading of Ni exhibited 89.2% of ABE conversion resulting in 79.9% of the total C_5–C_{15} yield. The results show that the availability of higher basic sites at MgO–Al_2O_3 plays a major role in the double alkylation of alcohols for the production of long-chain hydrocarbons. Further, the presence of Ni nanoparticles stimulates the dehydrogenation reaction, which significantly enhances the C_5–C_{15} conversion with 79.9% yield. The authors performed recyclability experiments that demonstrated 17.1% conversion drop due to the diminished surface area of the catalyst after four cycles.

4.4. Organo Amine Catalyzed Dimerization of Ketones

The higher oxygen content in LCB must be removed for its efficient conversion into high-energy-density fuels and chemicals. In contrast, improving the O/C ratio from biomass-derived fuels is essential for higher fuel generation. It can be achieved by catalyst-mediated aldol-type condensation, which substantially increases the O/C ratio for sustainable production of advanced fuel precursors. In 2015, Sankaranarayanapillai and co-workers reported a new approach using heterogeneous organic amine catalysts supported by silica–alumina (Si–Al) for aldol-type condensation for the selective dimerization of methyl ketones from LCB to generate liquid transport fuel precursors [113]. Among the screened organic amines, a Si–Al-assisted secondary amine maximized the dimerization of methyl ketone, which also displayed better water tolerance over primary and tertiary amines. Moreover, the yield of ketone dimers with C_4–C_{15} exceeded 60% of the other tested amines.

5. Integrated Catalytic Approaches for Advanced Biofuels Production

Previous reports on chemical catalysts involved in the generation of advanced biofuels are primarily based on simulations mimicking ABE mixtures, which are not necessarily representations of actual ABE fermentation broths used for advanced biofuel upgradation. For example, the catalysts utilized for simulated ABE bioconversion do not have tolerance for native ABE fermentation broth, which contains 95 wt.% of water [114]. Hence, various factors need to be optimized; especially, the design of the catalyst needs to be improved by enhancing the selectivity towards alcohols over that for water [115]. Furthermore, enhanced production of ABE by biological methods, integrated with chemical catalysis as well as an efficient downstream process for the separation of fuel supports industrial applications (Figure 4). The first report about an integrated catalytic approach for converting ABE fermentation products into higher hydrocarbon ketones was proposed by Anbarasan et al. [101]. ABE produced from *Clostridium acetobutylicum* ATCC824 using renewable substrates was simultaneously extracted using a non-toxic and water-immiscible solvent, glyceryl tributyrate. Glycerol tributyrate aided in the in situ removal of acetone and butanol, with simultaneous removal of inhibitors from the fermentation broth. Further, the higher boiling point of glyceryl tributyrate also reduced the energy requirement during the distillation process, suggesting its feasibility in industrial applications. The effect of various transition metals (Ni, Ru, Rh, Pd, Ir, and Pt) in the conversion of the extractant phase of the ABE mixture into higher ketones was evaluated with K_3PO_4 in toluene using alkylating reactions. Among the tested metals, Pd/C-K_3PO_4 provided an excellent conversion of ABE mixture into ketones at optimal conditions (20 h, 145 °C, 1.28 M K_3PO_4). Under this controlled alkylation reaction, the molar yield of 86% higher hydrocarbon ketones was obtained from the ABE mixture, with the alkylation of acetone as the limiting step of the process. The extractive fermentation using glyceryl tributyrate helped overcome the catalysts' sensitivity to water, significantly improving the catalyst's recyclability. The dehydrogenation and aldol condensation (Guerbet reaction) of alkylation products obtained from the ABE mixture yielded higher alkanes which can be utilized as substitutive or alternative aviation fuels. However, the major setback in the process is the generation of lower yield of C_{11} fractions due to the alkylation of acetone. Hence, obtaining diesel range components (>C_{11} components) from the integrated catalytic strategies is necessary. Thus, modifying the catalyst supporting the condensation of ABE (Guerbet reaction) before alkylation with acetone can address this issue [116]. Sreekumar et al. [117] reported the increased production of higher-range diesel components using HT supported Pd and Cu catalysts. A modified Pd and Cu-supported catalyst with HT increased the selectivity of the diesel components (>C_{11}) range mixture using tailored biomass fermentation of the ABE mixture. The ABE mixture treated with Pd–HT and Cu–HT catalyst at 240 °C in toluene significantly increased the C_{11} concentration to 29.5% and 28.4% with overall yields of 95% and 92%, respectively. Thus, these modified catalysts actively participate in the dehydrogenation of alcohol to induce subsequent aldol condensation with acetone, which allows for the production of higher ketone blends at higher quantities. Further, the enhanced production of the biofuel blendstocks was significantly improved by integrating fed-batch fermentation of *Clostridium acetobutylicum* with in situ extraction and heterogeneous catalytic reaction [117]. After fermentation, the alcohols were extracted using an immiscible extractant tributyrin, with which the extractant phase was upgraded using a Pd-HT catalyst. The alkylation reaction performed at 250 °C in a high-pressure Q-tube reactor for 20 h obtained ~0.5 g of high-value ketone blends from 1.7 g of fermentation product with a total yield of 86%.

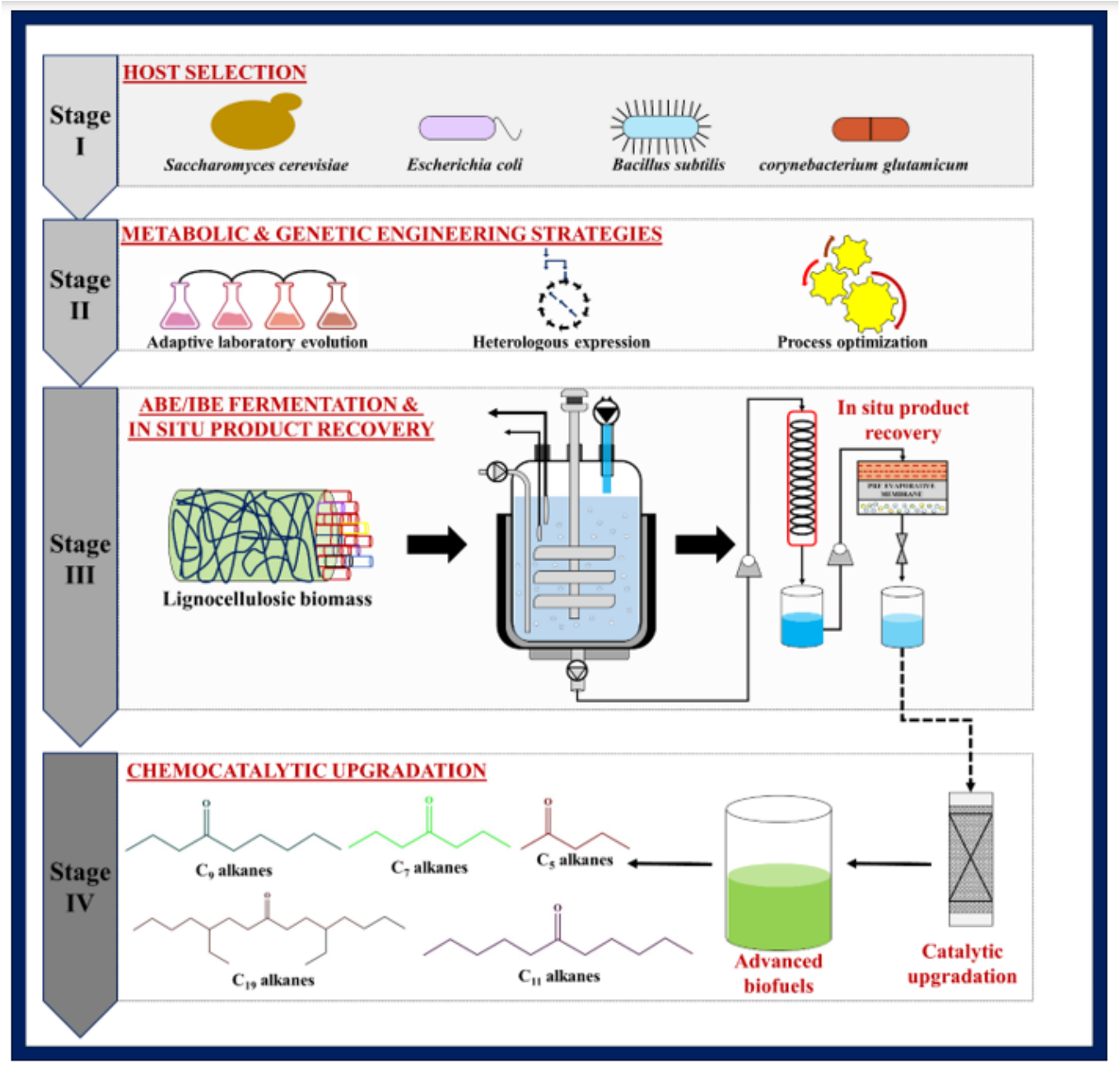

Figure 4. Integrated chemo and bio-catalytic approaches for advanced biofuel production from ABE fermentation.

The integrated techniques stated above have focused more on the extractant phase of the ABE mixture. However, the alkylation of the aqueous phase of the mixture with alcohol has not been explored in detail. Hence, identifying the optimal integration process with continuous in situ recovery of alcohol from the aqueous phase, with higher selectivity of alcohols over water eventually improved the sustainability of the process. Xue et al. [118] demonstrated the production of long-chain ketones from renewable agricultural residues by integrating chemical and bio-catalysis coupled with in situ gas stripping, which prevented the drying of ABE solvents ahead of alkylation. In situ gas stripping was performed by utilizing the fermentation off-gas (CO_2 and H_2) and the purity of ABE solvents was improved by pre-evaporation. Further, gas stripping improved ABE production by reducing the butanol toxicity to *Clostridium beijerinckii* CC101. This integrated approach reports the direct alkylation of ABE into long-chain ketones using Pd/C catalyst under continuous

mode. The two-stage in situ gas stripping produced a concentrated aqueous mixture of ABE (>500 g/L), which was simultaneously converted into longer chain ketones (C_5–C_{15}) with 70% conversion rate.

However, the efficient conversion of biomass-derived ABE to advanced biofuels is often restrained by the presence of 95 wt.% of water in the bulk fermentation broth. Besides water content, catalyst dosage, and alkalinity also play a critical role in industrial-scale production. Even though several methods have been reported for the concentration or separation of fuel precursors and ABE from the fermentation broth, viz., liquid–liquid extraction, gas-stripping, etc., all these methods require several distillation columns (at least three), which significantly increases the energy requirement and economy of the process. Hence, an alternative extraction process with reduced separation cycles to fractionate the fuel precursors from the fermentation broth is necessary for cost-effective, large-scale production of advanced biofuels. Recently, Xie et al. [119] reported a one-pot method for producing refined fuel precursors using Pd/C and separating these using K_3PO_4 salting-out process. In this study, the salting-out effect of K_3PO_4 was able to remove >99.5% water from the fermentation broth. Further, K_3PO_4 was involved in an alkylation reaction that resulted in the overall production of 82 wt.% of C_5–C_{11} compounds. Adapting this technique facilitated the omission of distillation units for the separation and purification of ABE from the fermentation broth.

6. Economic Considerations for Production of ABE-Based Advanced Biofuels

Thorough techno-economic analysis is the crux of the sustainable development of advanced biofuel products, especially since, at present, the cost of production of these biofuels is not competent enough compared to conventional fuels, thus limiting the possibility that they can replace conventional fuels. This has also led to widespread concerns among policymakers and stakeholders regarding the effectiveness of incentive programs [120]. It is crucial to examine multiple factors during economic analysis of advanced fuel production from ABE mixtures. The availability and cost of biomass feedstock, including non-food crops, lignocellulosic residues, and industrial waste streams significantly impact the overall economics of the process. Therefore, robust and adaptable value chains with sustainability measures and flexible protocols for feedstock sourcing are necessary to ensure continuous production while maximizing environmental benefits.

Other than feedstock, the scalability of the process also plays a crucial role in ensuring commercial viability. An evaluation of potential challenges and opportunities in scaling up the process from laboratory to industrial scale is mandatory to predict production costs. Policymakers acknowledge the importance of interventions, such as subsidies and tax benefits, in enhancing the cost competitiveness of advanced fuels while considering the negative economic and social impacts of fossil fuel imports and combustion. Incentives such as landfill taxes, higher carbon prices, tax breaks, subsidies, and increased taxes on fossil fuels can boost the advanced fuel industry [121]. Notably, a substantial increase in carbon costs across sectors would result in higher fossil fuel prices, creating a more favorable economic environment for these technologies [122].

7. Conclusions and Future Perspectives

Global transportation accounts for roughly 25% of total greenhouse gas emissions. Hence, decarbonizing this sector is essential to achieve the transition towards net zero carbon emissions as envisioned by the UN sustainable development goals. To achieve a reduction of the new carbon inflow into the environment, aviation fuels from renewable biomass should be considered. With the advancements made in bioprocess, genomic, and metabolic engineering, improved production of advanced biofuels can be achieved via two routes: (a) modifying the microbial host to tolerate higher biofuels in a cost-efficient process; and (b) the integration of metabolic and chemical catalysis for the upgradation of ABE to form higher biofuels, which have been outlined in significant detail in this review.

In the first route, further enhanced productivity can be achieved by expanding substrate utilization of improved strains to make it capable of utilizing a wide range of wastes, including persistent wastes (municipal and plastic wastes) for higher biofuel production, preferably in a harsh (non-sterile) environment. As an added benefit, the co-production of other value-added products with reduced downstream process steps can significantly improve the overall economy of the process.

The synergistic approach of the second route can significantly reduce the cost and steps involved in the purification of products generated from fermentation for advanced biofuel production. Furthermore, the synergy between metabolic engineering and chemical catalysis for the utilization of primary alcohols as advanced biofuels should be supported by stringent techno-economic and life-cycle assessment to ensure a smooth transition from the laboratory to the industrial scale, with a reduced carbon footprint.

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