

Review

Biorefinery: The Production of Isobutanol from Biomass Feedstocks

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Abstract: Environmental issues have prompted the vigorous development of biorefineries that use agricultural waste and other biomass feedstock as raw materials. However, most current biorefinery products are cellulosic ethanol. There is an urgent need for biorefineries to expand into new bioproducts. Isobutanol is an important bulk chemical with properties that are close to gasoline, making it a very promising biofuel. The use of microorganisms to produce isobutanol has been extensively studied, but there is still a considerable gap to achieving the industrial production of isobutanol from biomass. This review summarizes current metabolic engineering strategies that have been applied to biomass isobutanol production and recent advances in the production of isobutanol from different biomass feedstocks.

Keywords: isobutanol; biorefinery; metabolic engineering; biomass utilization

1. Introduction

Energy and the environment are two major issues facing the world. Due to climate change and the demand for renewable transportation fuels, the production of environmentally friendly biofuels has aroused great interest. Compared with fossil fuels, biofuels are more sustainable and highly renewable, which has attracted much attention [1–7].

In the past few years, researchers have focused on the production of biofuels from edible crops [8]. This is not a long-term solution. From the perspective of economy and sustainability, the ultimate goal is to convert low-cost non-edible biomass resources into high-value biofuels and other chemical products [9–13]. Therefore, people have proposed the biotransformation of lignocellulosic biomass, most of which is agricultural waste. With the development of biorefinery, the potential industrialization of lignocellulosic biomass processing to release monosaccharides that can be fermented and converted into high-value chemicals has become a reality. Cellulosic isobutanol already occupies a place in the fuel sector [14].

Ethanol has obvious disadvantages compared to fossil fuels, but most current research in biorefinery is still based on ethanol production. We need to expand the types of chemicals that can be produced to facilitate further development of biorefinery. Compared with ethanol, most higher alcohols have lower hygroscopicity, higher energy density, higher octane number, and properties that are closer to gasoline, so they have a higher compatibility with existing equipment and higher operating safety factors [15–17]. Isobutanol is an important industrial compound. It is used in lubricants, coatings, adhesives, automobile spray paint, and as an intermediate for the synthesis of many drugs. In addition, isobutanol derivatives are widely used in the chemical industry [15]. As a new generation of biofuel, isobutanol has many advantages (Table 1) and uses [18–20]. Therefore, developing of biorefinery to produce biomass isobutanol is in line with market needs and sustainable development.

Table 1. Major characteristics comparison of several common biofuels with gasoline.

	Ethanol	1-butanol	Isobutanol	Gasoline
Lower Heating Value (MJ/kg)	27.0	33.1	33.3	43.5
Flash point (°C)	13	37	28	−43
Solubility (20 °C in water, wt %)	Miscible	7.7	8.7	negligible
Boiling temperature (°C)	78.4	117.7	108	25–215
Vapor toxicity	Toxic	Moderate	Moderate	Moderate

Biorefinery involves many steps of biomass treatment, biomass conversion, fermentation, product purification, fermentation processes, etc. In this article, we discuss the industrial microorganisms used in biorefinery, review the metabolic engineering strategies of isobutanol production by microorganisms, and introduce the current situation and developmental prospects of isobutanol production from various biomass feedstocks.

2. Research on Isobutanol Production

The isobutanol synthesis pathway (Figure 1) starts when pyruvate is converted to 2-ketoisovalerate (KIV) by acetolactate synthase (AHAS), acetoxyacid reductoisomerase (AHARI), and dihydroxyacid dehydratase (DHAD), respectively. Next, KIV is converted to isobutyraldehyde by the 2-ketoacid decarboxylase (KIVD), and finally alcohol dehydrogenase (ADH) produces isobutanol (we call the pathway consisting of these five enzymes the engineered isobutanol pathway below). Microorganisms that are widely used to biosynthesize isobutanol include *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. Most microorganisms do not produce, or only produce trace amounts of isobutanol on their own. Now several strategies can increase isobutanol production, such as the overexpression of key enzymes of the isobutanol synthesis pathway, the inhibition of byproduct production, cofactor engineering, and microbial robustness enhancement. We selected some representative studies to briefly introduce current microbial isobutanol production strategies.

In 2008, Liao's group [16] validated the potential of microbes to produce higher alcohols using *E. coli*. By introducing KIVD from *Lactococcus lactis* and ADH from *S. cerevisiae*, the engineered strain JCL260 successfully produced isobutanol using glucose as a substrate. Then increasing the pyruvate and 2-ketoacid concentrations, JCL260 produced 22 g/L of isobutanol, which, at 86% of the theoretical maximum, demonstrated this strategy's potential. This is the first time that researchers have used metabolic engineering strategies to produce isobutanol with microorganisms. The cytotoxicity of isobutanol causes high concentrations of isobutanol to inhibit cell growth, thus limiting the maximum titer. Liao's group [21] further combined an in situ product removal strategy (gas stripping) with isobutanol production to improve the final titer of isobutanol. The final isobutanol production reached 50 g/L after 72 h of fed-batch fermentation in a bioreactor. They also used the chemical mutagen N'-nitro-N-nitrosoguanidine (NTG) to induced sequential mutagenesis in *E. coli* and screened for a high-yielding isobutanol strain. Repairing an inhibitory mutation resulted in a final isobutanol titer of 21.2 g/L isobutanol in 99 h [22]. Another factor that limits the isobutanol titer is a cofactor imbalance. Arnold's group [23] removed the dependence of the isobutanol synthesis pathway on NAPDH in *E. coli* and achieved 100% of the theoretical maximum isobutanol production during growth in anaerobic conditions.

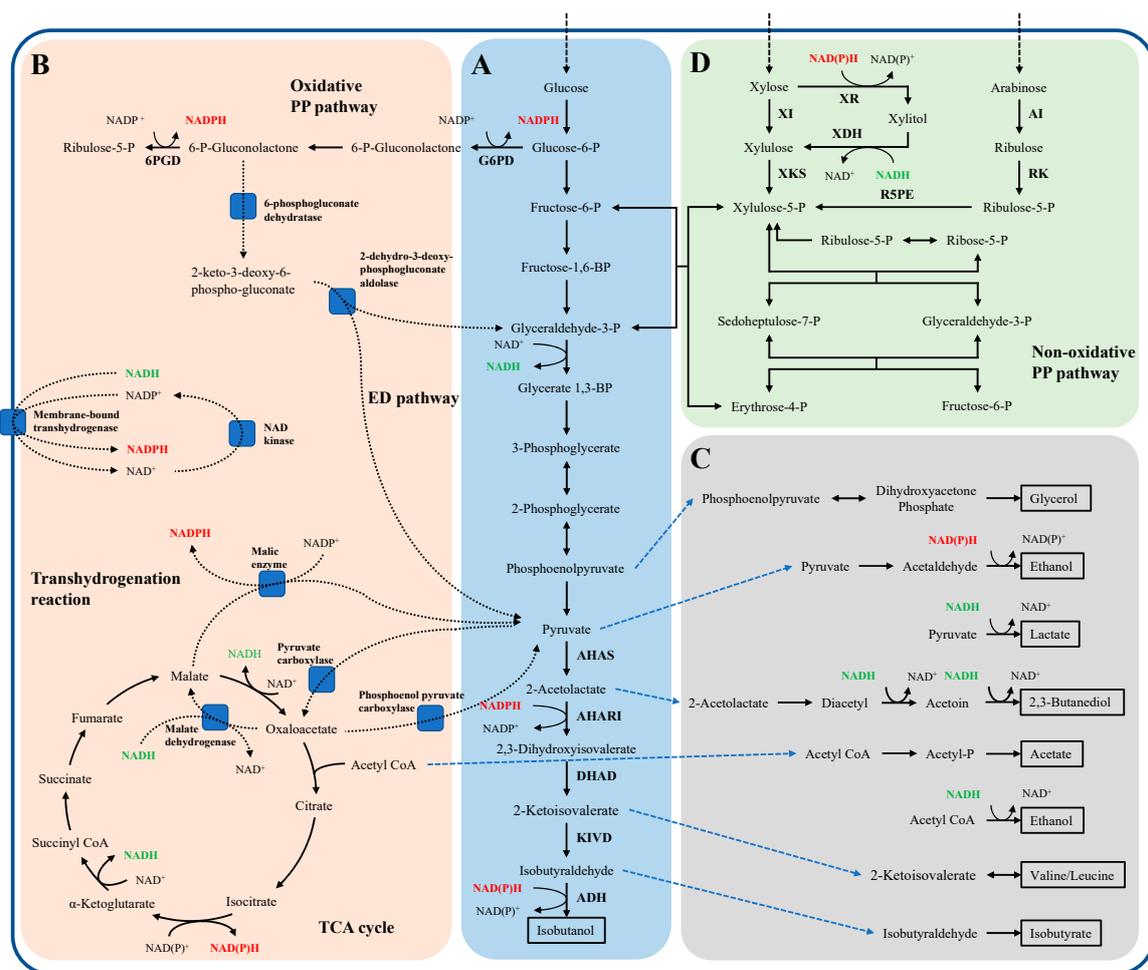


Figure 1. Schematic diagram of microorganism isobutanol synthesis pathways. Part (A) shows the general pathways of isobutanol biosynthesis; Part (B) shows a partial cofactor balancing strategy for successful optimization of isobutanol synthesis; Part (C) shows the common by-product synthesis pathways of isobutanol synthesis; and Part (D) shows the sugar metabolism pathway using non-glucose fermentation to produce isobutanol. The dashed line indicates the source of the precursor substance for the byproduct; AHAS: Acetolactate synthase; AHARI: Acetohydroxyacid reductoisomerase; DHAD: Dihydroxyacid dehydratase; KIVD: Keto acid decarboxylase; ADH: Alcohol dehydrogenase; G6PD: Glucose-6-phosphate dehydrogenase; 6PGD: 6-phosphogluconate dehydrogenase; XR: Xylose reductase; XDH: Xylitol dehydrogenase; XI: Xylose isomerase; XKS: Xylulokinase; AI: L-arabinose isomerase; RK: L-Ribulokinase; R5PE: L-Ribulose-5-P-4-epimerase.

C. glutamicum is widely used to produce various amino acids, so some researchers have explored isobutanol production by *C. glutamicum*. In 2010, Liao's group [24] tried to produce isobutanol with *C. glutamicum* for the first time. They overexpressed the engineered isobutanol pathway, and on this basis deleted the *PYC* gene (encoding pyruvate carboxylase) and *LDH* gene (encoding lactate dehydrogenase). This engineered strain they obtained produced 4.9 g/L of isobutanol when fermented for 96 h. Blombach's group [25] found that the main byproducts of *C. glutamicum* during isobutanol production are lactic and succinate. By knocking out the byproduct synthesis genes, restoring the redox balance in combination with heterologous expression of transhydrogenase (*pntAB* from *E. coli*), and overexpressing *adhA*, the final isobutanol titer reached 13 g/L at 48 h. Recently, Inui's group [26] has used different promoter combinations to confirm the importance of higher activity of AHAS and KDC for isobutanol synthesis in *C. glutamicum*. In combination with a cofactor strategy (altering the cofactor specificity of AHARI and ADH) and enhanced glycolytic flux strategy (overexpression of endogenous glycolytic genes and the phosphoenolpyruvate:carbohydrate phosphotransferase system

(PTS), plus introduction of the Entner–Doudoroff pathway from *Zymomonas mobilis*), 20.8 g/L isobutanol was produced at 24 h to reach 84% of the theoretical value.

Wen's group [27] proved that *B. subtilis* could be used as a cell factory for isobutanol synthesis through an isobutanol tolerance test. In 2011, they introduced the engineered isobutanol pathway into *B. subtilis*, resulting in strain BSUL03. The concentration of the isobutanol reached 2.63 g/L at 54 h. Next, they performed an elementary mode analysis (EMA) on the engineered strain BSUL03 to identify targets in the metabolic network that could be optimized, lactate dehydrogenase and pyruvate dehydrogenase complexes. By knocking out the *ldh* and *pdhC* genes, they engineered the strain BSUL05 and obtained an isobutanol titer of 5.5 g/L after 60 h of fermentation [28]. Afterwards, they performed a metabolic flux analysis and comparison of the two engineered strains. To increase the concentration of NADPH and achieve a redox balance, the engineered strain BSUL08 was obtained by knocking out *pgi* (encoding glucose 6-phosphate isomerase), overexpressing *zwf* (encoding glucose 6-phosphate dehydrogenase), and further overexpression the transhydrogenase gene (*udhA* from *E. coli*). The production performance was tested with fed-batch fermentation and the isobutanol titer reached 6.12 g/L at 60 h, which was 63% of the theoretical maximum [29].

S. cerevisiae can produce a small amount of higher alcohols through the biosynthetic pathway of various amino acids. It also has a natural tolerance to alcohols that is higher than other microorganisms [30]. In recent years, *S. cerevisiae* has been widely used to produce higher alcohols. In 2011, Chen's group [31] overexpressed of the genes *ILV2* (encoding acetolactate synthase), *ILV3* (encoding dihydroxy acid dehydratase), and *ILV5* (encoding diacetolactate reductase) to obtain an isobutanol yield of 3.86 mg/g glucose. This is the first record of *S. cerevisiae* being used to produce isobutanol. Based on that, Boles's group [32] truncated the N-terminal mitochondrial targeting sequence of the *ILVs* to achieve expression of *ILV2*, *ILV3*, and *ILV5* in the cytoplasm, and optimized the codons of these three genes. They studied the activities of KIVD and ADH and determined that *ARO10* and *ADH2* were the most active enzymes in the synthesis of isobutanol. The final concentration of isobutanol produced by the strain they obtained was 0.63 g/L. Then they [33] knocked out the synthesis pathway of leucine, isoleucine, 2,3-butanediol, glycerol, pantothenate, and isobutyrate, thereby increasing the carbon flux of the isobutanol metabolism pathway. Here, the isobutanol titer reached 2.09 g/L in 96 h. Ethanol has always been the largest byproduct of isobutanol production, but the complete removal of pyruvate decarboxylase activity would also arrest cell growth. Therefore, Avalos's group [34] designed two powerful optogenetic gene expression systems in *S. cerevisiae*. In the presence of light, this system induces the expression of *PDCs* and promotes cell growth; in the dark, it induces the expression of *ILV2* to enhance the isobutanol biosynthesis. By controlling light exposure during fermentation, they could control ethanol production and could increase the isobutanol titer to 8.49 g/L.

3. Biomass Isobutanol Production

3.1. Isobutanol Production from Lignocellulose

Isobutanol is considered a promising alternative to gasoline, and cellulosic isobutanol is becoming increasingly important in the wave of next-generation biofuels. Extensive research in cellulosic ethanol [35,36] and cellulosic butanol [37,38] has provided a theoretical basis sufficient for the synthesis of cellulosic isobutanol. Some researchers have carried out feasibility analyses on the industrial production of cellulose isobutanol [39,40], confirming the great potential of cellulose isobutanol. However, few studies focus on the synthesis of isobutanol from lignocellulose biomass (details are summarized in Table 2).

3.1.1. Cellulosic Isobutanol Produced by Natural Cellulose-Degrading Microorganisms

Degrading cellulose with microorganisms that naturally utilize lignocellulose and convert the resulting C5 and C6 sugars into isobutanol is the simplest way to obtain cellulosic isobutanol. To minimize the lignocellulose glycation process and production costs, the consolidated bioprocessing

(CBP) strategy was developed. The first cellulosic isobutanol was synthesized by introducing the engineered isobutanol pathway into *Clostridium cellulolyticum* [41], which utilizes cellulose naturally. The strain produced a final isobutanol titer of 0.66 g/L. The simple introduction of the isobutanol synthesis pathway resulted in very low amounts of isobutanol titer, which is not sufficient for industrial scale production. The inability of *C. cellulolyticum* to process large amounts of substrate has been reported [42]. Destroying the ability of *C. cellulolyticum* to form spores by knocking out the *spo0A* gene improved cellulose utilization, but there was little variation in isobutanol titer (even lower than the wild type when the cellulose substrate reaches 50 g/L) [43]. The biosynthesis of cellulose isobutanol was also attempted in *Geobacillus thermoglucosidasius* [44], which produced isobutanol at 0.6 g/L from cellobiose in 60 h. While unsuccessful at producing large volumes of cellulosic isobutanol, this experiment demonstrated the strong thermal stability of ALAS and KIVD, which are widely used for isobutanol synthesis.

Table 2. Summary of microbial utilization of biomass to produce isobutanol.

Microorganism	Carbon Source	Strategy	Genes Involved	Titer	Time	Reactor	Reference
<i>Clostridium cellulolyticum</i>	Cellulose	Engineered isobutanol pathway	<i>ilvD_{EC}</i> , <i>ilvC_{EC}</i> , <i>yqhD_{EC}</i> , <i>alsS_{BS}</i> , <i>kivd_{LL}</i>	0.66 g/L	216 h	Tube	[41]
	Cellulose	Keto acid pathway Promoter engineering	Δ <i>spo0A</i> , <i>alsS_{BS}</i> , <i>kivd_{LL}</i>	0.35 g/L	~250 h	Unknown	[43]
<i>Geobacillus thermoglucosidasius</i>	Cellobiose	Keto acid pathway Promoter engineering	<i>ilvC_{CT}</i> <i>alsS_{BS}</i> , <i>kivd_{LL}</i> (LLKF_1386)	0.6 g/L	48 h	Tube	[44]
<i>Clostridium thermocellum</i>	Cellulose	Keto acid pathway Promoter engineering Optimize fermentation conditions	<i>ilvB_{CT}</i> , <i>ilvN_{CT}</i> , <i>ilvC_{CT}</i> , <i>ilvD_{CT}</i> , <i>kivd_{LL}</i>	5.4 g/L	75 h	Tube	[45]
	Cellulose	Inhibition competition pathway Adaptive laboratory evolution	Δ <i>hpt</i> , Δ <i>ldh</i> , Δ <i>pta</i> , <i>adhE^{D494G}</i>	5.1 g/L	220 h	Bioreactor	[46]
<i>Trichoderma reesei</i> and <i>Escherichia coli</i>	Pretreated corn stover	Random mutagenesis	<i>T. reesei</i> RUTC30: -	1.88 g/L	380 h	Bioreactor	[47]
		Engineered isobutanol pathway Microbial consortium	<i>E. coli</i> NV3: <i>ilvC_{EC}</i> , <i>ilvD_{EC}</i> , <i>alsS_{BS}</i> , <i>kivd_{LL}</i> , <i>Adh2_{SC}</i>				
<i>Caldicellulosiruptor bescii</i>	Switchgrass	Inhibition competition pathway AOR-ADH pathway	Δ <i>ldh</i> <i>PF0346_{PF}</i> (AOR), <i>Teth514_0564_{PF}</i> (ADHA)	0.17 g/L	40 h	Fermentor	[48]
	Glucose-xylose mixture	Dismantle carbon catabolite repression Inhibition competition pathway Engineered isobutanol pathway	Δ <i>ldhA</i> , Δ <i>adhE</i> , Δ <i>pflB</i> , Δ <i>pta-ackA</i> , <i>mlc*</i> , <i>ilvC_{EC}</i> , <i>ilvD_{EC}</i> , <i>alsS_{BS}</i> , <i>kivd_{LL}</i> , <i>Adh2_{SC}</i> ,	11 g/L	182 h	Flask	[49]
	Cedar	Dismantle carbon catabolite repression Inhibition competition pathway Promoter engineering Chromosome integration Optimize fermentation conditions	Δ <i>ldhA</i> , Δ <i>adhE</i> , Δ <i>pflB</i> , Δ <i>pta-ackA</i> , <i>mlc*</i> , <i>ilvC_{EC}</i> , <i>ilvD_{EC}</i> , <i>alsS_{BS}</i> , <i>kivd_{LL}</i> , <i>adhA_{LL}</i>	3.7 g/L	96 h	Flask	[49,50]

Table 2. Cont.

Microorganism	Carbon Source	Strategy	Genes Involved	Titer	Time	Reactor	Reference
<i>Saccharomyces cerevisiae</i>	Xylose	Xylose XI pathway Cytosolic isobutanol pathway	$\Delta Ilv2$, $\Delta Ilv5$, $\Delta Ilv3$, $xylA_{CP}$, $Tal1_{SC}$, $Xks1_{SC}$, $Ilv2\Delta N54_{SC}$, $Ilv5\Delta N48_{SC}$, $Ilv3\Delta N19_{SC}$, $Aro10_{SC}$, $Adh2_{SC}$	1.36 mg/L	150 h	Flask	[51]
	Xylose	Xylose XI pathway Chromosome integration Adaptive laboratory evolution Mitochondrial isobutanol pathway Fed-batch fermentation	$\Delta BAT1$, $\Delta ALD6$, $\Delta PHO13$, $\Delta URA3$ $RKI1_{SC}$, $RPE1_{SC}$, $TKL1_{SC}$, $TAL1_{SS}$, $XYLA_{PE}$, $XYL3_{SS}$, $ILV2_{SC}$, $ILV5_{SC}$, $ILV3_{SC}$, $kivd_{LL}$, $AdhA_{LL}^{RE1}$	3.1 g/L	192 h	Tube	[52,53]
	Xylose	Xylose XR-XDH pathway Chromosome integration Mitochondrial isobutanol pathway Copy number optimization Adaptive laboratory evolution	$\Delta PHO13$, $\Delta GRE3$, $hxt7^{F79S}$, $XYL1_{SS}$, $XYL2_{SS}$, $XYL3_{SS}$ $ILV2_{SC}$, $ILV5_{SC}$, $ILV3_{SC}$, $ADH7_{SC}$, $kivd_{LL}$	92.9 mg/L	144 h	Tube	[53,54]
	Xylose	Xylose XR-XDH pathway Chromosome integration Copy number optimization Mitochondrial isobutanol pathway Optimize fermentation conditions Fed-batch fermentation	$\Delta ALD6$, $\Delta PHO13$ $XYL1_{SS}$, $XYL2_{SS}$, $XYL3_{SS}$ $ILV2_{SC}$, $ILV5_{SC}$, $ILV3_{SC}$, $kivd_{LL}$, $AdhA_{LL}^{RE1}$	2.6 g/L	Unknown	Bioreactor	[55,56]
<i>Corynebacterium glutamicum</i>	Hemicellulose fraction	Inhibition competition pathway Xylose XI pathway Arabinose metabolism pathway Engineered isobutanol pathway	Δpqo , $\Delta ilvE$, $\Delta ldhA$, Δmdh , $xylA_{XC}$, $xylB_{CG}$, $araB_{EC}$, $araA_{EC}$, $araD_{EC}$, $ilvB_{EC}$, $ilvN_{EC}$, $ilvC_{EC}$, $ilvD_{EC}$, $pntA_{BEC}$, $kivd_{LL}$, $Adh2_{CG}$	0.53 g/L	~28 h	Flask	[57,58]

Table 2. Cont.

Microorganism	Carbon Source	Strategy	Genes Involved	Titer	Time	Reactor	Reference
	Cellobiose	Inhibition competition pathway Copy number optimization Engineered isobutanol pathway Cellobiose metabolism pathway	$\Delta adhE, \Delta frdBC, \Delta fur, \Delta ldhA, \Delta pta, \Delta pflB, ilvC_{EC}, ilvD_{EC}, alsS_{BS}, kivd_{LL}, adhA_{LL}, bglCTF$	7.64 g/L	72 h	Unknown	[59]
	Cellobionic	Inhibition competition pathway Engineered isobutanol pathway	$\Delta adhE, \Delta frdBC, \Delta fur, \Delta ldhA, \Delta pta, \Delta pflB, ilvC_{EC}, ilvD_{EC}, alsS_{BS}, kivd_{LL}, adhA_{LL}$	1.4 g/L	48 h	Unknown	[60]
<i>Corynebacterium crenatum</i>	Duckweed	Engineered isobutanol pathway	$ILV2_{SC}, ILV5_{SC}, ILV3_{SC}, kivd_{LL}, Adh2_{SC}$	1.15 g/L	96 h	Flask	[61]
	Duckweed	Whole-cell mutagenesis Engineered isobutanol pathway Simultaneous saccharification and fermentation	$ILV2^*_{SC}, ILV5^*_{SC}, ILV3_{SC}, kivd_{LL}, Adh6^*_{SC}$	5.6 g/L	96 h	Flask	[62]
	Emptyfruit bunches	Engineered isobutanol pathway Optimize fermentation conditions Separate hydrolysis and fermentation	$ilvC_{EC}, ilvD_{EC}, adhP_{EC}, alsS_{BS}, kivD_{LL}$	5.4 g/L	156 h	Unknown	[62]
<i>Enterobacter aerogenes</i>	Sugarcane bagasse	Inhibition competition pathway Engineered isobutanol pathway Pervaporation-coupled fermentation	$\Delta ldhA, \Delta budA, \Delta pflB, \Delta ptsG, ilvD_{KP}, ilvC_{KP}, budB_{KP}, kivD_{LL}, adhA_{LL}$	23 g/L	72 h	Fermenter	[63,64]
<i>Escherichia coli</i>	Algal protein	Chemical mutagenesis Protein conversion Cofactor engineering	$\Delta glnA, \Delta gdhA, \Delta luxS, \Delta lsrA, ilvC^{A71S, R76D, S78D, Q110A}, yqhD^{G39I, S40R}, ilvE_{EC}, ilvA_{EC}, sdab_{EC}, avta_{EC}, LueDH_{TI}, ilvD_{EC}, alsS_{BS}, kivD_{LL}$	0.2 g/L	Unknown	Flask	[65,66]

Table 2. Cont.

Microorganism	Carbon Source	Strategy	Genes Involved	Titer	Time	Reactor	Reference
<i>E. coli</i> BLF2 and <i>E. coli</i> AY3 (1:1.5)	Distillers' grains	Chemical mutagenesis Protein conversion Cofactor engineering Engineered isobutanol pathway Microbial consortium	<i>E. coli</i> BLF2: Δ <i>ldh</i> <i>ilvC</i> _{EC} , <i>ilvD</i> _{EC} , <i>YqhD</i> _{EC} , <i>alsS</i> _{BS} , <i>ktivD</i> _{LL} <i>E. coli</i> AY3: Δ <i>glnA</i> , Δ <i>gdhA</i> , Δ <i>luxS</i> , Δ <i>lsrA</i> , <i>ilvC</i> ^{A71S, R76D, S78D, Q110A} , <i>yqhD</i> ^{G39I, S40R} <i>ilvE</i> _{EC} , <i>ilvA</i> _{EC} , <i>sdab</i> _{EC} , <i>avta</i> _{EC} , <i>LueDH</i> _{TI} <i>ilvD</i> _{EC} , <i>alsS</i> _{BS} , <i>ktivD</i> _{LL}	6.5 g/L	52 h	Tube	[65,67]
<i>Bacillus subtilis</i>	Okara wastes	Activation of <i>ilv-leu</i> operon Inhibition competition pathway Keto acid pathway	Δ <i>codY</i> , Δ <i>bkdB</i> , Δ <i>relA</i> , <i>LueDH</i> _{TI} , <i>ktivD</i> _{LL} , <i>yqhD</i> _{EC}	0.02 g/L	Unknown	Flask	[68]
<i>Bacillus subtilis</i> and <i>Escherichia coli</i> (1:4)	Watermelon rind and Okara waste	Protein conversion Engineered isobutanol pathway Microbial consortium	<i>B. subtilis</i> : Δ <i>codY</i> , Δ <i>bkdB</i> , <i>LueDH</i> _{TI} , <i>ktivD</i> _{LL} , <i>yqhD</i> _{EC} <i>E. coli</i> : <i>ilvC</i> _{EC} , <i>ilvD</i> _{EC} , <i>YqhD</i> _{EC} , <i>alsS</i> _{BS} , <i>ktivD</i> _{LL}	0.88 g/L	220 h	Flask	[69–72]

Note: The abbreviation in the upper left corner of the gene indicates that the gene is a mutation; abbreviations in the lower right corner of genes indicate microorganisms of gene origin. BS: *Bacillus subtilis*; CG: *Corynebacterium glutamicum*; CP: *Clostridium phytofermentans*; CT: *Clostridium thermocellum*; EC: *Escherichia coli*; GT: *Geobacillus thermoglucosidasius*; KP: *K. pneumoniae* KCTC2242; LL: *Lactococcus lactis*; PE: *Piromyces* sp. E2; PF: *Pyrococcus furiosus*; PS: *Pichia stipites*; SC: *Saccharomyces cerevisiae*; SS: *Scheffersomyces stipitis*; TF: *Thermobifida fusca*; TI: *Thermoactinomyces intermedius*; XC: *Xanthomonas campestris*.

The cellulose-utilizing *Clostridium thermocellum* is considered a promising producer of cellulosic biofuels, so additional experiments have attempted cellulosic isobutanol synthesis in this bacterial species [69]. Refinement gene expression systems [70–72] and gene editing techniques [73] have already been developed for *C. thermocellum*, and its basic metabolic network has been explored. Introducing the KIVD into *C. thermocellum*, optimizing the isobutanol synthesis pathway, and limiting the urea content of the medium enabled the engineered strain to ferment 5.4 g/L of isobutanol in 75 h [45]. Experiments have also identified a new pathway in *C. thermocellum* that converts KIV to isobutanol. This pathway decarboxylates KIV to isobutyryl-CoA with a ketoisovalerate ferrous-dependent reductase (KOR), which is converted to isobutanol by the aldehyde/alcohol dehydrogenase (ADH). Most recently, Holwerda's group [46] engineered *C. thermocellum* by eliminating the acetic and lactic synthesis pathways and performed adaptive laboratory evolution, resulting in the strain LL1043 that produced an isobutanol titer of 5.1 g/L. High titer were obtained without optimizing isobutanol synthesis, suggesting that the production of cellulose isobutanol with *C. thermocellum* remains a possibility.

Some new studies attempt to apply isobutanol production to other microbes. Lin's group [47] designed a microbial consortium that cocultured the cellulose utilizing strain *Trichoderma reesei* with an isobutanol-producing strain of *E. coli* to produce 1.88 g/L of isobutanol from pretreated corn stover. Another study [48] investigated a new AOR-ADH pathway for alcohol production. This pathway converts acetate to ethanol via aldehyde ferredoxin oxidoreductase (AOR) and ADH. The cellulolytic extreme thermophile *Caldicellulosiruptor bescii* naturally produces acetate. Heterologous expression of the AOR-ADH pathway in *C. bescii*, resulted in ethanol synthesis. Isobutanol was synthesized using switchgrass as a carbon source following isobutyrate supplementation, providing new ideas for subsequent isobutanol synthesis.

3.1.2. Cellulosic Isobutanol Produced by Non-Native Cellulose-Degrading Microorganisms

Another strategy for obtaining cellulosic isobutanol is to further reform the pre-existing engineered strains for isobutanol production to utilize lignocellulose or its pretreatment products. *E. coli* and *S. cerevisiae* are the most promising cellulosic isobutanol-producing strains. Two model organisms have invested considerable research in cellulose utilization, which have been summarized in a considerable number of reviews [74,75]. They both have significant carbon catabolite repression and cannot utilize glucose simultaneously with other sugars. Xylose is the most abundant sugar in lignocellulose aside from glucose, so many efforts have been to construct industrially viable xylose-utilizing strains. To eliminate carbon catabolite repression, *E. coli* used UV mutagenized screened a new target gene [49], *Mlc*, encoding a DNA-binding transcriptional repressor. A strain with this mutant *Mlc* gene outperformed previous research in fermenting isobutanol from mixed glucose and xylose. Further optimization of the isobutanol synthesis pathway genes expression (*ilvC*, *ilvD*, *alsS*, *kivd* and *adhA*) with the biomass-inducible chromosome-based expression system (BICES) [50], achieved an isobutanol titer of 3.7 g/L from cedar hydrolysate.

S. cerevisiae cannot convert xylose naturally, but considerable efforts have been made to engineer a strain of *S. cerevisiae* that can [76]. There are two types of xylose metabolism (Figure 1D), one catalyzed by xylose reductase (XR) and xylitol dehydrogenase (XDH), the other by xylose isomerase (XI). However, the XR-XDH pathway requires the cofactor NADPH that is also in high demand for isobutanol synthesis, prone to cofactor imbalance. For this reason, the XI pathway is favored for isobutanol synthesis, but most attempts to express heterologous xylose isomerases in *S. cerevisiae* have failed. XylA, a xylose isomerase from *Clostridium phytofermentans*, was successfully expressed in *S. cerevisiae* by Boles' group [51]. Further, they increased xylose metabolism by overexpressing *Xks1* and *Tal1*, with the redesigned cytoplasmic isobutanol pathway, it achieved first isobutanol production by using xylose as the sole carbon source. Stephanopoulos' group engineered a strain to produce ethanol from xylose by the introducing xylose isomerase from *Piromyces sp. E2* in *S. cerevisiae*. Overexpressing *RK11*, *RPE1*, *TKL1*, *TAL1* and *XYL3* in this background increased xylose assimilation and facilitated adaptive laboratory evolution [52]. Boles' group [53] further optimized the use of

xylose in this strain by knocking out gene *PHO13*. With expression of the mitochondrial isobutanol pathway and inhibition of valine and acetic acid synthesis, the production of isobutanol was 3.1 g/L in 192 h of fed-batch fermentation. These experiments show that xylose promotes mitochondrial activity significantly more than glucose does and increases with increasing concentrations of xylose. Thus, the advantages of isobutanol production through the mitochondrial isobutanol pathway from xylose were demonstrated. Another xylose utilization pathway, the XR-XDH pathway, has also been used to produce isobutanol. Runguphan's group [77] determined through combinatorial screening that the xylose metabolism pathway from *Scheffersomyces stipitis* is most effective when applied to isobutanol synthesis. Incorporating the strategies identified in previous studies to enhance xylose conversion and assimilation (knockout *PHO13*, *GRE3* and overexpression of *XYL3*) and optimizing the copy number of isobutanol pathway genes produced 48.4 mg/L of isobutanol within 144 h. Adaptive laboratory evolution of the resulting engineered strain, and identification of two newly discovered mutation targets (the *CCR4*^{A638S} and *TIF*^{A79S}) to improve xylose utilization [54] increased the xylose ratio growth rate by 40.6%, but had little impact on isobutanol production. Using the XR-XDH pathway is likely to cause redox imbalance, and numerous studies have attempted to alleviate the cofactor imbalance by altering the cofactor specificity of the enzyme [78–80]. Jin's group [55] adjusted the copy numbers of three genes (*XYL1*, *XYL2*, and *XYL3*) in *S. cerevisiae* to improve redox balance and reduce acetate and xylitol accumulation. These results of evolutionary engineering confirmed the importance of deleting *PHO13* for xylose utilization. The final isobutanol titer was 2.6 g/L when coupled with the mitochondrial isobutanol pathway [56]. Metabolite analysis of the engineered strain revealed that the use of xylose increased valine levels in *S. cerevisiae*, which can be converted by branched-chain amino acid transaminases into KIV. This once again confirms that xylose promotes isobutanol synthesis.

C. glutamicum is also a promising industrial producer of isobutanol, and it is necessary to develop an engineered strain that can utilize cellulose. By heterologous expression the xylose XI and the arabinose metabolic pathways from *E. coli*, Blombach's group [57] succeeded in constructing strains that can rapidly utilize mixed sugars, including glucose. When combined with previous strategies to optimize isobutanol synthesis [25,58], 0.53 g/L of isobutanol could be produced from hemicellulose fractions.

One way to address the fermentation of mixed sugars containing xylose is to bypass the use of glucose. Cellobiose is an intermediate product of cellulose hydrolysis to glucose and has no carbon catabolite repression effects on cells. Mixed sugar fermentation using cellobiose and xylose has been reported [81,82], but no studies have addressed isobutanol synthesis. Isobutanol production using cellobiose was attempted based on a previously engineered strain from *E. coli*. Expression of β -glucosidase from *Thermobifida fusca* resulted in an isobutanol production of 7.64 g/L by optimizing gene copy number [59]. It should be noted that direct transport of cellobiose to intracellular hydrolysis is required to avoid carbon catabolite repression, and no growth of cells expressing the cytoplasmic enzyme was observed in this study. Another study [60] tested cellobionic acid, the main product of cellulose hydrolysis assisted by the use of lytic polysaccharide monoxygenase (LPMO), as a carbon source for the production of isobutanol. *E. coli* was found a naturally utilize the cellobionic acid pathway. A final titer of 1.4 g/L was produced from the cellulose hydrolysate of *Neurospora crassa* conversion, thus expanding the available carbon source for cellulosic isobutanol.

Most of the studies described above are based on either industrially produced cellulose or the monosaccharides obtained from its hydrolysis as substrates, but there is a lack of studies on the direct use of cellulose or its hydrolysates to produce isobutanol. Depending on the source of cellulosic biomass, the proportions of cellulose, hemicellulose, and lignin can vary considerably [83]. The hydrolysis products will also be significantly different, depending on how the pretreatment is metabolized [84]. These hydrolysis products (weak acids, furan derivatives, phenolic compounds, etc.) toxic to cells and reduce the isobutanol titer. Several experiments have tested the ability of engineered isobutanol-producing strains designed to ferment the hydrolysis products of cellulosic biomass. Duckweed [61,85] is a fast-growing non-food crop with few growth requirements that is very easy to handle. Empty fruit bunches [62] are a large byproduct of palm oil production and

sugarcane bagasse [63] is a common industrial byproduct. Their pretreated fermentation inhibitor content is low, as can be concluded from the comparison with glucose fermentation. This resulted in these biomasses being good carbon source providers. The best isobutanol titer was obtained from *Enterobacter aerogenes* [64] use of sugarcane bagasse hydrolysates to produce 23 g/L of isobutanol by pervaporation-coupled fermentation.

3.2. Isobutanol Production from Protein

Biorefinery is focused on the industrial production of high-value compounds from biomass. Industrial waste like distillers' grains and okara waste cannot be integrated into the most existing strategies due to their primary component, proteins. For the process of protein hydrolysis to amino acids, some amino acids produce 2-keto acids, which are precursors of isobutanol. Liao's group [65,86] has pioneered the use of proteins as feedstock for higher alcohols production. In *E. coli*, they used chemical mutagenesis to screen the *YH19* strain that can utilize 13 amino acids as a sole carbon source. The restriction of ammonia assimilation, introduction of three exogenous transamines and deamines cycles, and knocking out population-sensing genes (*luxS* and *lsrA*) generated the strain *YH83* [65]. Strain *YH83* expresses the engineered isobutanol pathway that used algal protein hydrolysates to produce higher alcohols. Taking the more severe cofactor imbalance triggered by the use of protein biomass into consideration, Davis' group [66] performed cofactor-specific reconstructions of 2,3-dihydroxy isovalerate oxidoreductase (IlvC) and NADPH-dependent isobutanol dehydrogenase (YqhD) in the *YH83* strain to obtain an isobutanol titer of 0.2 g/L from algae protein hydrolysate. Davis' group [67] then attempted to utilize both sugars and proteins from biomass by constructing an *E. coli* microbial consortium, that is coculturing a proportional mix of strain *AY3*, which can produce heteroalcohols from proteins, and strain *BL2*, which can produce isobutanol from glucose and xylose. Protein utilization increased from 16.3% to 31.3% in a separate culture at a ratio of 1.5:1. The fermentation of distillers' grains hydrolysate under these conditions also resulted in the highest isobutanol titer of 6.5 g/L, which was higher than the 5.5 g/L achieved with strain *BL2* fermentation alone. The fermentation of other biomass like algal protein hydrolysates was also investigated and the highest isobutanol titer of 2.38 g/L was achieved when strains *AY3* and *BL2* were fermented in a 4:1 ratio, demonstrating the industrial potential of this microbial consortium.

Since *E. coli* does not naturally produce the proteases required to process protein biomass, further attempts were made in *B. subtilis*, which secretes proteases that enable growth on polypeptides. Due to the different genetic backgrounds of *E. coli* and *B. subtilis*, a series of experiments were performed to determine the importance of *codY* (a global regulator) deletion for inhibiting ammonia assimilation and improving branched-chain amino acid synthesis. Combined with a strategy of preventing the degradation of branched-chain amino acid by knocking out the dihydrolipoyl acyltransferase (*bkdb*) in the branched-chain 2-keto acid dehydrogenase complex, a final biofuel titer of 0.72 g/L was obtained from protein biomass [86]. Based on this, Choi's group [68] knocked out *RelA*, expressing the regulatory protein responsible, that recognizes nutritional stress to activate the *ilv-leu* operon [87] and further promotes branched-chain amino acids synthesis. Comparing the results of spent coffee grounds and okara wastes fermentation, the triple deletion strain expressing *KIVD* and *ADH* produced almost no isobutanol with spent coffee grounds hydrolysate as a substrate, while okara wastes hydrolysate fermented about 0.02 g/L of isobutanol in eight days. The microbial consortium strategy was also applied to this engineered strain and isobutanol was successfully obtained from the hydrolysates of watermelon rind and soybean residue by coculturing with *E. coli* *AY3* [88]. The highest isobutanol titer was 0.88 g/L at an *E. coli* to *Bacillus subtilis* ratio of 4:1. From these studies that used proteins to produce isobutanol, it was found that differences in the source of biomass could lead to large changes in product ratios, which require further research.

4. Conclusions

In recent years, researchers have made considerable effort to increase the yield of microbial biofuel production and continued to explore chemical synthesis from different biomass materials [13]. In addition, some achievements have been made in the production of bioethanol [14,89]. In this paper we summarized current metabolic engineering strategies applied to biomass isobutanol production and recent advances in the production of isobutanol from different biomass feedstocks.

It can be seen that in the last decade, there was a significant improvement in the production of cellulosic isobutanol while the synthesis of isobutanol from protein biomass was demonstrated. Biomass isobutanol is no longer at the theoretical stage. However, the efforts made in the broader context of biorefining were not enough. Since isobutanol is a secondary metabolite, controlling the competing pathway to maximize the conversion of pyruvate to isobutanol is difficult, and removing the largest competitor, the ethanol pathway, can lead to severe growth defects. Coupled with the fact that biomass utilization efficiencies are low, the combination of the two problems leads to very low yields. Efficient inhibition of ethanol synthesis has been accomplished using light-controlled genetic systems [34], but the in vivo metabolic backgrounds of different microorganisms are very different, and further investment in research is needed to accomplish an efficient inhibition of the competing pathways. Another issue is how to enhance the robustness of engineered microorganisms, as both isobutanol and organics from biomass hydrolysates can cause considerable cellular damage. This issue includes how to improve the redox imbalance caused by isobutanol production, as it has been shown an improved redox significantly increases strain isobutanol tolerance [90] or furfural tolerance [91–93] and thus isobutanol production. Adaptive laboratory evolutionary applications for tolerance enhancement have obtained some results, with a large number of target genes being identified. However, the tolerance mechanisms are still opaque for both isobutanol and lignocellulose-derived microbial inhibitors. Research on the application of tolerance enhancement strategies to biomass isobutanol production is also quite scarce.

In particular, it should be noted that of all the current studies on the production of isobutanol from biomass, the highest titers reach 23 g/L by pervaporation-coupled fermentation, while the highest titers of isobutanol production are currently over 50 g/L due to in situ product removal. The timely isolation of isobutanol from the medium reduces the inhibitory effect of isobutanol on cells, reduces cytotoxicity, and significantly enhances the final titer. Fermentation processes also have a considerable influence on the production of isobutanol, but the existing research on microbial isobutanol synthesis and product separation [92,93] is weakly linked. This aspect also needs to be strengthened in future research on microbial isobutanol production. We believe that the synthesis of biomass isobutanol will make significant progress with the further research on these issues.

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