



Review

# A Short Review of Second-Generation Isobutanol Production by SHF and SSF

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**Abstract:** As isobutanol exhibits higher energy density and lower hygroscopicity than ethanol, it is considered a better candidate biofuel. The sustainable supply of inedible biomass and lack of competition with the food supply have stimulated significant worldwide interest in the production of isobutanol from this resource. Both separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) have been applied to isobutanol production to effectively utilize inedible biomass as a feedstock. However, both processes have various challenges, including low isobutanol yield and high production costs. This review summarizes the potential of isobutanol as a biofuel, methods for conferring isobutanol productivity, recent experimental studies, and developments in both SHF and SSF with the isobutanol-producing strains. Challenges to increasing the isobutanol yield and various suggestions for improvements to enable commercial production are also discussed.

**Keywords:** isobutanol; biomass utilization; separate hydrolysis and fermentation; simultaneous saccharification and fermentation; metabolic engineering



**Citation:** Akita, H.; Matsushika, A. A Short Review of Second-Generation Isobutanol Production by SHF and SSF. *Appl. Biosci.* **2024**, *3*, 296–309. <https://doi.org/10.3390/applbiosci3030020>

Academic Editors: Robert Henry, Nicolai S. Panikov and Nikolaos Kourkoumelis

Received: 14 May 2024

Revised: 30 June 2024

Accepted: 2 July 2024

Published: 8 July 2024



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## 1. Introduction

As populations increase, the worldwide economy continues to develop, creating greater demands for energy. Most of the energy demand is currently met by the use of fossil fuels, such as coal, oil, and natural gas. However, as supplies of fossil fuels are limited, eventual depletion is inevitable. Moreover, the increased utilization of fossil fuels has significantly impacted the environment. Global warming due to fossil fuel consumption represents one of the greatest threats to worldwide economic sustainability, as climate change has been linked to extreme weather events, sea level rise, and changes in climate patterns, all of which negatively impact biodiversity [1,2]. In the face of these challenges, biofuels are receiving increased attention as potential alternative energy sources that could contribute to the mitigation of global warming. As biofuels can be produced from renewable resources such as biomass, their use has the potential to significantly reduce greenhouse gas emissions. Moreover, the use of biofuels in industry is increasing because this reduces fossil fuel consumption and thereby suppresses the progression of global warming.

Biofuels have been used since the beginning of the automotive industry. In the 1940s, biofuels were considered practical fuels for transportation, and several types were used in the United States, Germany, and the United Kingdom, such as Agol (up to 17% bioethanol), Monopoline (up to 30% bioethanol), and Cleveland Diskol (up to 30% bioethanol), respectively [3]. After World War II, biofuels research and development stagnated due to low fossil fuel prices, but many countries began showing renewed interest in biofuels for commercial use amid the oil crisis of the 1970s. In the late 1990s, as oil prices and energy security concerns increased, many countries, including the United States, developed policies to support the domestic biofuel industry [4]. In addition, Brazil began commercial production

of bioethanol as part of its national program [5]. Currently, more than 60 countries have instituted national programs for biofuel production, including the Renewable Fuel Standard in the United States [6] and the Renewable Energy Directive in Europe [7], which set targets for blending biofuels into the national fuel supply [8]. Based on the resulting policies, global bioethanol production increased approximately 16.5-fold, from 6.7 billion liters to 110.4 billion liters, in the decade from 2008 to 2018 [9]. Similarly, biodiesel production increased approximately 3.4-fold, from 12 billion liters to 41 billion liters, over this time frame [9]. In addition, to ensure the stability of jet fuel supplies, airlines utilize biofuels prepared from several feedstocks (Table 1). To date, biofuels account for approximately 3.4% of total transportation fuels worldwide [9].

**Table 1.** Utilization of biofuels for jet fuel. \* Data include paid, airlift, and test flights.

Year	Airline *	Primary Feedstock	Mixture Ratio (%)	Reference
2007	Air New Zealand	Jatropha	50	[10]
2008	Virgin Atlantic	Coconut, babassu	20	[11]
2009	Continental Airlines	Algae, jatropha	50	[12]
	Japan Airlines	Algae, jatropha, camelina	50	[13]
	KLM Royal Dutch Airlines	Camelina	50	[14]
2010	United Airlines	Algae	N/A	[15]
	Aeromexico	N/A	N/A	[16]
	LATAM Airlines Brasil	Jatropha	50	[17]
2011	Air China	Jatropha	50	[18]
	Air France	Waste cooking oil	N/A	[19]
	InterJet	Jatropha	27	[20]
	KLM Royal Dutch Airlines	Waste cooking oil	20	[21]
2012	All Nippon Airways	Waste cooking oil	N/A	[22]
	Etihad Airways	Waste cooking oil	N/A	[23]
	Nippon Cargo Airlines	Waste cooking oil	N/A	[24]
	United Airlines	Algae	40	[25]
2014	Finnair	Waste cooking oil	N/A	[26]
	Lufthansa Airlines	Farnesene sugar	100	[27]
2016	Alaska Airlines	Forest residual	20	[28]
	United Airlines	Algae	N/A	[29]
2017	Singapore Airlines	Palm oil	N/A	[30]
2018	SpiceJet	Cooking oil, seeds of oil-bearing plants	N/A	[31]
2020	American Airlines	Waste nonedible oil, waste wood	N/A	[32]
	Etihad Airways	Waste cooking oil	50	[23]
	Singapore Airlines	N/A	N/A	[30]
2021	All Nippon Airways	Algae	N/A	[33]
	Etihad Airways	Waste cooking oil	38	[23]
	Japan Airlines	Algae, discarded clothes, wood chips	N/A	[34]
	United Airlines	Algae	100	[35]
2022	Fuji Dream Airline	Algae, waste cooking oil	N/A	[36]
	Lufthansa	Waste cooking oil	N/A	[37]
	Malaysia Airlines	Animal fat waste	N/A	[38]
2023	Qantas	Agricultural waste, waste cooking oil, energy crops	N/A	[39]
	AirAsia	Agricultural residual	N/A	[40]
	Emirates	Plant sugar, waste fats	100	[41]
	Finnair	Agricultural waste, waste cooking oil	N/A	[42]
	Cathay Pacific Airlines	Animal fats, waste cooking oil	N/A	[43]
	Virgin Atlantic Airways	Plant sugar, waste fats	100	[44]
	Vistara	Jatropha	17	[45]

Based on the feedstocks and procedures used in production, biofuels can be classified up to the third generation (Table 2). First-generation biofuels are produced from edible biomass sources such as starch or sugars. Corn is the most common feedstock utilized in the United States [46], whereas sugarcane is the primary feedstock used in Brazil [47]. In Europe, potatoes, wheat, and sugar beets are commonly used as feedstock [48]. As

the abovementioned feedstocks require little or no pretreatment, they are often used for bioethanol production. Currently, more than 130 billion liters of bioethanol are produced annually [49] and mixed with gasoline or diesel oil [8]. For example, E10, defined as gasoline containing 10% fuel-grade ethanol, is used as regular gasoline in the United States and Brazil [50]. However, the production of first-generation biofuels is encountering increasing criticism for its competition with the human food supply due to hunger and other food-related challenges around the world. As such, second-generation biofuels are now being produced from inedible biomass for which there is no competition with the food supply. Second-generation biofuels are produced from inedible biomass such as lignocellulose and food residuals, and technical development and demonstration projects aimed at commercializing the process are underway in many countries.

**Table 2.** Benefits and limitations of each generation of biofuel.

Producer Organism	Feedstock	Benefits	Limitations
<b>First-generation</b> Yeast	Edible biomass (starch, sugar, etc.)	Simple pretreatment process High production yield	Competition with food supply
<b>Second-generation</b> Yeast or bacteria	Inedible biomass (lignocellulose, food residual, etc.)	Abundant carbon resources No competition with the food supply	Complex pretreatment process Low production yield
<b>Third-generation</b> Algae	Carbon dioxide	No competition with the food supply More than 10-fold the carbon dioxide fixation capacity of higher plants Simple production process	Challenges in mass-production technology Low production yield

Compared with ethanol, isobutanol has a higher octane number and exhibits nearly 30% greater volumetric energy density (Table 3). By contrast, isobutanol is less soluble in water; therefore, isobutanol–gasoline mixtures may be less susceptible to phase separation. Thus, isobutanol is considered a better gasoline additive or substitute candidate than ethanol. In addition, isobutanol is also a useful building block. For example, isobutylene, which is synthesized by the dehydration of isobutanol, is used as a raw material for jet fuel, plastics, rubber, lubricants, and other hydrocarbons [51]. According to SkyQuest Technology, the isobutanol market was valued at USD 1.24 billion in 2022 and is expected to grow to USD 2.01 billion in 2030 with a CAGR of 6.2% during 2023–2030 [52]. The global isobutanol market is established by industries such as chemicals, automotive, and paints, which consume isobutanol produced from fossil fuels. On the other hand, in recent years, environmental considerations and sustainability become paramount, increasing the demand for isobutanol produced from renewable feedstocks in those industries.

**Table 3.** Comparison of fuel characteristics. The data are taken from Reference [53].

Characteristic	Isobutanol	Ethanol	Gasoline
Research octane number	106	110	88–98
Motor octane number	90	90	80–88
Boiling point (°C)	108	78	27–225
Flash point (°C)	28	13	7.6
Autoignition temperature (°C)	415	363	–43
Energy density (MJ/L)	26.6	21.4	30–33
Reid vapor pressure (kPa)	3.3	16	54–103
Lower heating value (MJ/kg)	33.1	26.8	41–44
Heat of evaporation (MJ/kg)	0.69	0.92	0.36
Lower flammability limit concentration (vol%)	1.7	3.3	0.37–0.44
Upper flammability limit concentration (vol%)	11.8	19	1.4

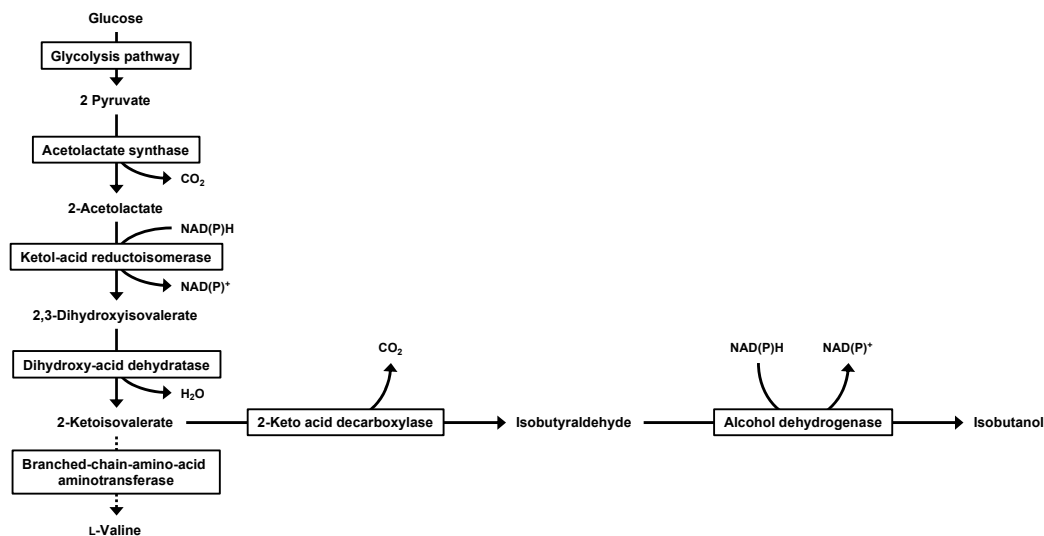
Despite the advantages of using isobutanol produced from inedible biomass, several challenges remain, including high production costs. Several isobutanol production methods, such as separate hydrolysis and fermentation (SHF) as well as simultaneous

saccharification and fermentation (SSF), using inedible biomass as a feedstock have been reported recently to overcome these limitations. However, a comprehensive review summarizing both processes has not been reported. Thus, this review aims to identify research gaps necessary to achieve the industrial application by summarizing the recent progress and challenges of SHF and SSF. Section 2 describes the development of isobutanol-producing strains and their productivity. Sections 3 and 4 provide overviews of SHF and SSF and summarize the challenges of each process. Section 5 summarizes the advantages and disadvantages of SHF and SSF and discusses the solutions to increase isobutanol productivity. The paper ends with conclusions and recommendations for establishing an economical process for isobutanol production from inedible biomass.

## 2. Development of Isobutanol Production Capacity

The advantage of using microorganisms for isobutanol production is the capacity to perform a multistep production process in a single cell. Another advantage is that isobutanol can be produced from sugars without requiring high temperature and pressure conditions. In contrast to ethanol and *n*-butanol production, wild-type microorganisms cannot produce large amounts of isobutanol [54]. Thus, to enable the production of isobutanol, artificial pathways must be engineered into wild-type microorganisms. Microbial production of a large amount of isobutanol was first demonstrated using *Escherichia coli* [55]. In this case, the artificial pathway was constructed by integrating heterologous genes into the native Ehrlich and valine biosynthesis pathways, with the pyruvate produced by glycolysis used as a starting material (Figure 1). In the initial step, two molecules of pyruvate are converted to 2-acetolactate by acetolactate synthase (encoded by *alsS*). Subsequently, 2-acetolactate is converted to 2,3-dihydroxyvalerate by ketol-acid reductoisomerase (encoded by *ilvC*), which is then converted to 2-ketoisovalerate by dihydroxy-acid dehydratase (encoded by *ilvD*). Finally, 2-ketoisovalerate is converted to isobutyraldehyde by 2-keto acid decarboxylase (encoded by *kivd*); isobutyraldehyde is further converted to isobutanol by alcohol dehydrogenase (encoded by *adh2*). This pathway can be used to produce a large amount of isobutanol by employing two highly active enzymes: acetolactate synthase from *Bacillus subtilis* and 2-keto acid decarboxylase from *Lactococcus lactis*. Although acetolactate synthase (encoded by *ilvIH*) is conserved as the native enzyme in *E. coli*, 2-ketobutyrate is preferred over pyruvate, and the activity is lower than that of the enzyme from *B. subtilis*. Thus, acetolactate synthase from *B. subtilis* is utilized. By contrast, the activity of 2-keto acid decarboxylase from *L. lactis* is 2.5-fold higher than that of other 2-keto acid decarboxylases. A major advantage of this method is that it enables the production of isobutanol from glucose. The current production strategy has been extended to various genera of bacteria (including *Bacillus*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Geobacillus*, *Pseudomonas*, *Ralstonia*, and *Synechococcus*) and yeast (including *Saccharomyces* and *Pichia*). *E. coli* is often used as a host due to its superior growth capacity, well-understood metabolic pathway, and availability of suitable genetic recombination technologies.

In microbial production, the production yield never reaches 100% because sugars are used for cell growth in addition to isobutanol production. Thus, efficient isobutanol production is important. To achieve efficient isobutanol production, it is necessary to eliminate cofactor imbalance. Among the enzymes forming the isobutanol biosynthesis pathway, ketol-acid reductoisomerase and alcohol dehydrogenase utilize NADPH as a cofactor. In the *E. coli* cell, under aerobic conditions, NADPH is produced by three major pathways: pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA), and membrane-bound transhydrogenase [56]. To reduce the amount of by-products and increase the production yield, isobutanol production is preferably carried out under anaerobic conditions. However, PPP and TCA cycle do not function under anaerobic conditions, and NADH is mainly produced by glycolysis under anaerobic conditions. Thus, the earlier study used aerobic conditions for isobutanol production [55]. Now, cofactor imbalance has been resolved by using both enzymes modified to utilize NADH [57,58], with modified cofactor specificity, allowing anaerobic production of isobutanol.



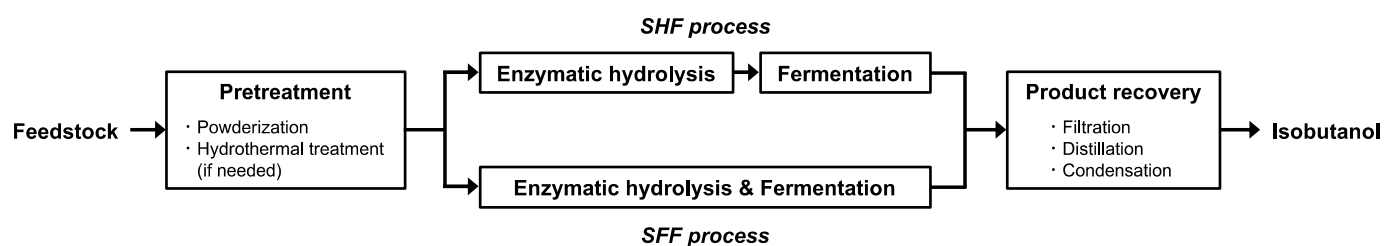
**Figure 1.** Artificial pathway for isobutanol production in *E. coli*.

The highest concentration of isobutanol reportedly obtained in previous studies in which *E. coli* JCL260 was incubated in a synthetic medium based on glucose was 50.8 g/L [59], and *E. coli* JCL260 also exhibits the highest productivity, at 0.706 g/(L·h) [59]. These values are approximately 2-fold higher than those obtained in previous studies. It is thought that the use of batch fermentation led to the excellent isobutanol concentration and productivity obtained in this case. Batch fermentation is often used for high-density culture of microorganisms. If nutrients necessary for high-density culture are contained in the broth before inoculation, microbial growth will be inhibited by osmotic effects and high-concentration inhibition, and this is the case even for nutrients that do not normally inhibit growth. By contrast, osmotic effects and high-concentration inhibition can be avoided if nutrients are added gradually at a low rate, thus enabling a successful high-density culture. However, various challenges must be overcome for commercial production using many existing methods, including methods based on *E. coli* JCL260. For example, these methods require expensive reagents, such as expression plasmids and gene expression inducers, to promote the expression of heterologous genes necessary for isobutanol production by host cells, and this leads to increased production costs. Moreover, synthetic medium prepared using highly purified reagents or edible biomass are not suitable for commercial production due to the high cost associated with the large volume of medium required. In these methods, isobutanol was produced using a small-scale jar fermenter (up to 1 L), but a bench-scale study is necessary to ultimately enable industrial-scale production. Thus, to date, research has focused on the use of inedible biomass as a feedstock for isobutanol production.

### 3. Second-Generation Isobutanol Production by SHF

The use of inedible biomass as a feedstock for isobutanol production generally involves a multistep process consisting of pretreatment, enzymatic hydrolysis, and fermentation (Figure 2). The pretreatment process is essential for enhancing sugar yields from inedible biomass and is divided into four categories: physical, chemical, physicochemical, and biological methods [60]. In particular, the physical method with hydrothermal pretreatment is a cost-effective method for the degradation of the rigid structure of lignocellulose. In the physical method, lignocellulose is mechanically milled to prepare particles with a diameter of approximately 1.0 mm. Subsequently, for easy access to saccharification enzymes during enzymatic hydrolysis, the resulting particles are hydrothermally treated to degrade into cellulose, hemicellulose, and lignin. Cellulose is composed of hexose glucose, whereas hemicellulose is composed of pentoses, such as xylose and arabinose. These polysaccharides are hydrolyzed into monosaccharides using cellulases and hemicellulases. Thus, it is important to increase the yield of the glucose polymer cellulose; that is, increasing

the yield of cellulose will lead to an increase in the final production yield of isobutanol. Since the ionic product of water increases from  $10^{-14}$  to  $10^{-11}$  as the temperature increases up to 250 °C, water is considered to act as an acid catalyst [61]. Moreover, hydrothermal pretreatment using water does not require the addition of harmful chemicals or expensive non-corrosive metals and therefore has less environmental impact than other methods. Another advantage of hydrothermal pretreatment using water can be observed using oil palm empty fruit bunches. Hydrothermal treatment of oil palm empty fruit bunches at 180–200 °C leads to a decrease in hemicellulose yield with increasing temperature, and the glucose yield is increased due to inhibition of xylose, mannose, galactose, and arabinose generation [62]. Based on its superior advantages, hydrothermal treatment is used to prepare hydrolysates from sugarcane bagasse [63], Japanese cedar [64], and empty fruit bunch [65].



**Figure 2.** SHF and SFF for second-generation isobutanol production.

Enzymatic hydrolysis is necessary to hydrolyze the cellulose and hemicellulose present in hydrothermally treated samples in order to produce primarily glucose and xylose. Enzymatic hydrolysis involves incubating hydrothermally treated samples with saccharification enzymes under conditions optimized for those enzymes. Several different saccharification enzymes that exhibit a broad range of activities are commercially available [66]. As such, different saccharification enzymes must be used depending on the target sample. A mixture of saccharification enzymes such as *Acremonium* cellulase, Optimash BG, and Novozyme 188 is often used for the hydrothermal treatment of samples prepared from lignocellulose. *Acremonium* cellulase and Novozyme 188, which catalyze the hydrolysis of cellobiose to produce bimolecular glucose, exhibit particularly high  $\beta$ -glucosidase activity of any commercially available enzymes. By contrast, Optimash BG, which is a mixture of  $\beta$ -glucanase and xylanase, hydrolyzes cellobiose, and hemicellulose. Thus, using a mixture of these enzymes is highly effective for extracting glucose and xylose from hydrothermally treated samples, as these sugars are preferable carbon sources for the microorganisms. On the other hand, as Cellulase ONOZUKA R-10 and Macerozyme R-10 exhibit hemicellulase and pectinase activities, a mixture of both enzymes is useful for extracting glucose and xylose from hydrothermally treated samples prepared from inedible biomass such as banana peel [66].

Isobutanol is produced during the fermentation step by incubating the hydrolysate with genetically engineered microorganisms. In general, the isobutanol concentration and productivity are lower when using hydrolysate compared with using a synthetic medium. The decreases in these parameters are caused by inhibitors produced as by-products during hydrothermal hydrolysis of cellulose and hemicellulose. For example, the aldehyde inhibitors furfural and 5-hydroxymethylfurfural are commonly detected in hydrolysates prepared from lignocellulose [67], and these compounds disrupt the cell membrane, inhibit enzymes, and cause DNA damage in microbial cells [68]. Organic acids that significantly inhibit microbial growth are also generated, such as acetate, formate, and glycolate. Thus, while batch fermentation as described above is an effective means of improving isobutanol productivity, it is also an effective means of imparting the capacity to detoxify inhibitors and thereby improve the growth of microorganisms in the hydrolysate. The detoxification capacity can be provided by the expression of aldehyde-degrading enzymes in the cell. For example, NADPH-dependent aldehyde reductase (KmGRE2) from *Kluyveromyces marxianus* strain DMB1 exhibits a broad substrate specificity

for aldehydes, and the KmGRE2-overexpressing *E. coli* showed improved growth with increased degradation of furfural when cultivated in cedar hydrolysate, as compared to cells not expressing the enzyme [69]. Moreover, overexpression of transcription factors may enhance the detoxification capacity of isobutanol-producing strains, since it has been demonstrated that overexpression of STB5 or ETP1 in yeast enhances the degradation of furfural contained in lignocellulosic hydrolysates and increases ethanol production [70].

Incubation of *Enterobacter aerogenes* EHM02 in hydrolysate prepared from sugarcane bagasse produced more than 23.0 g/L isobutanol, more than 4-fold higher concentration than those produced using hydrolysates prepared from switchgrass, Japanese cedar, cellulose, and empty fruit bunches (Table 4). This high production concentration results from several innovations. The first is the release of carbon catabolite repression (CCR). When mixed sugars are fed to microorganisms, glucose is preferentially utilized while other sugars remain unutilized, which is known as CCR. In bacterial glucose catabolism, glucose-specific permease PtsG is one of the proteins that function in the CCR. In *E. aerogenes* EHM02, the simultaneous consumption of glucose and xylose is enabled by the disruption of *ptsG*, which is effective for consuming mixed sugars in hydrolysate [63]. The second is the removal of isobutanol using the pervaporation membrane. The accumulation of isobutanol inhibits both the growth and enzymatic activity of *E. aerogenes* EHM02. To relieve the toxic effects of isobutanol on *E. aerogenes* EHM02, the pervaporation membrane is coupled to the bioreactor, which improves growth and isobutanol productivity [63].

**Table 4.** Comparison of isobutanol concentration and productivity obtained using inedible biomass as feedstock. N.D. means not described.

Strain	Feedstock	Concentration (g/L)	Productivity [(g/(L·h))]	Yield (%)	Reference
<b>SHF</b>					
<i>E. aerogenes</i> EHM02	Sugarcane bagasse	23.0	0.319	14	[63]
<i>Saccharomyces cerevisiae</i> HRW253	Switchgrass	5.52	0.115	24	[71]
<i>E. coli</i> mlcXT7-LAFC-AAKCD	Japanese cedar	3.70	0.0386	14	[64]
<i>E. coli</i> AL17	Cellulose	1.40	0.0300	36	[72]
<i>E. coli</i> JK209	Empty fruit bunch	1.40	0.0167	26	[65]
<i>Pichia pastoris</i> PPY0311	Sugarcane trash	0.0482	0.000669	N.D.	[73]
<b>SSF</b>					
<i>Corynebacterium crenatum</i> MA11C	Duckweed	5.61	0.0688	25	[74]
<i>E. coli</i> mlcXT7-LAFC-AAKCD	Banana peel	1.27	0.0148	N.D.	[66]
<i>S. cerevisiae</i> YEZ546-2	Cellulose	0.364	0.00758	N.D.	[75]

#### 4. Second-Generation Isobutanol Production by SSF

The concept of SSF was first proposed by Gauss et al. in 1976 [76]. They found that the final product produced by enzymatic hydrolysis inhibits the activity of saccharification enzymes and demonstrated that SSF improves ethanol yield by avoiding enzyme inhibition. This superior process is now commonly used in the production of various biofuels.

To our knowledge, there have been no reports of direct production of isobutanol from inedible biomass without pretreatment and enzymatic hydrolysis. In particular, enzymatic hydrolysis is an important process responsible for the extraction of carbon sources for isobutanol production. During enzymatic hydrolysis in SHF, the saccharification enzymes are generally first added to hydrothermally treated samples, and then the enzymatic reaction is allowed to proceed for several days, yielding a hydrolysate. Subsequent fermentation to produce isobutanol also proceeds for several days. Because at least one week is required to produce isobutanol using SHF, production costs are higher than when using chemical synthesis. SSF avoids glucose-mediated inhibition of cellulases and maintains fermentation inhibitors at low levels, thereby increasing the saccharification rate and yield of desired products, in contrast to SHF [77,78]. Wingren et al. reported that capital costs are comparable to the feedstock costs in ethanol production from lignocellulose, and simplifi-

cation of the production process by integrating SSF is estimated to reduce production costs by approximately 20% [78]. Thus, SSF has received increased attention for commercial second-generation isobutanol production because it enables enzymatic saccharification and fermentation for production in a single vessel.

Production of isobutanol by SSF has been reported using four inedible biomass sources to date, and in all cases, the isobutanol production time was shortened (Table 4). In this process, *C. crenatum* MA11C showed the maximum concentration of 5.61 g/L, which is more than 4.4-fold higher than the concentrations obtained in other studies. In the *C. crenatum* MA11C, by optimizing metabolic pathways using genetic mutations through undirected whole-cell mutagenesis, isobutanol productivity was enhanced by the accumulation of the precursor such as 2-ketoisovalerate [74].

To achieve effective isobutanol production using SSF, several factors must be considered. First, the reaction between saccharification enzymes and microorganisms should be maintained. For example, the highest concentration of isobutanol using duckweed as the feedstock was obtained by selecting saccharification enzymes that are active at the optimum pH and temperature for the isobutanol-producing strain, *C. crenatum* MA11C [74]. Similarly, Akita et al. enhanced isobutanol productivity by optimizing the enzyme activity within the pH and temperature ranges for optimal growth of the isobutanol-producing *E. coli* strain [66]. Second, the supplemental nutrients produced from the degradation of the feedstock enhance the growth of isobutanol-producing strains. In addition to glucose, treatment of duckweed by enzymatic hydrolysis produces galacturonic acid polysaccharides such as pectin [74]. Banana peel contains polysaccharides, proteins, and minerals [66]. Effective utilization of these nutrients can promote microbial growth and enhance isobutanol productivity. Third, the use of feedstock with high lignin content should be avoided to prevent growth inhibition of isobutanol-producing strains. Lignin generically consists of *p*-hydroxybenzene, guaiacyl (4-alkyl-2-methoxyphenol), and syringyl (4-alkyl-2,5-dimethoxyphenol) units, which are cross-linked by C-C bonds (e.g., 5-5,  $\beta$ -1,  $\beta$ -5,  $\beta$ - $\beta$ ) and C-O-C bonds (e.g., 4-O-5,  $\alpha$ -O-4,  $\beta$ -O-4) [79,80]. Thus, the degradation of lignin releases phenolic compounds that inhibit microbial growth.

A comparison of SSF and SHF in terms of the concentration and productivity of isobutanol reveals that both values are lower when using SSF (Table 4). These decreases are the result of differences in optimal pH between the saccharification enzymes and microorganisms. Depending on the purpose, several different commercial saccharification enzymes are commercially available, most of which are derived from fungi [65]. Fungal saccharification enzymes exhibit maximum activity at approximately pH 5.0–6.0 and a temperature of 40–50 °C. However, as isobutanol-producing strains are generally mesophilic, most strains cannot grow at temperatures more than 40 °C. The growth of these strains is also inhibited at acidic pH, which leads to a decrease in isobutanol productivity. Thus, eliminating differences in optimal pH is necessary to enhance the isobutanol concentration and productivity. In ethanol production by SSF using inedible biomass as a feedstock, productivity can reportedly be increased by using heat-resistant microorganisms [81]. Moreover, transcriptome analyses provided preliminary data regarding the molecular mechanism of stress tolerance in ethanol-producing strains, and this information can be useful in planning modification strategies using these strains [82]. It is possible to improve isobutanol productivity by applying the abovementioned techniques to isobutanol-producing strains.

## 5. Future Perspectives

Isobutanol produced by microbial fermentation using edible biomass such as sugar has already been commercialized by the US company Gevo [83]. Gevo established the world's first facility for the fermentation of isobutanol in Minnesota and achieved an annual isobutanol production of 750,000 to 1 million gallons in 2016. Isobutanol can be utilized as a gasoline additive, and the use of jet fuel chemically synthesized from isobutanol is increasing, primarily in Europe [83]. Thus, isobutanol has shown significant potential as a biofuel, second only to ethanol in utilization. As mentioned above, to avoid using



edible biomass as feedstocks, the commercialization of isobutanol production from inedible biomass has been widely studied around the world.

To date, two processes, SHF and SSF, have been developed as processes to effectively utilize inedible biomass as a feedstock for isobutanol production (Table 5). In SHF, enzymatic hydrolysis and fermentation are carried out separately, which enables each process to proceed under optimal conditions. Thus, it can avoid a mismatch of optimal conditions between enzymatic hydrolysis and fermentation, resulting in higher productivity. However, production time is longer due to the multistep process. By contrast, compared with SHF, SSF has several advantages, such as a simpler and easier-to-perform production process and reduced energy input, since enzymatic hydrolysis and fermentation are performed simultaneously. Moreover, the simplification of the production process reduces production time, which is directly related to production costs. Thus, we consider that SSF could be a cost-effective process to produce isobutanol if the productivity can be increased. Both processes can produce isobutanol from renewable feedstocks under ordinary temperature and pressure and do not produce harmful by-products. Thus, SHF and SSF are considered environmentally friendly production processes. However, in both processes, it should be noted that the downstream separation and purification steps have a significant impact on total production costs. Incidentally, it remains controversial whether SHF or SSF is more suitable for industrial application, and no conclusion has been reached even for ethanol, which has been researched more extensively than isobutanol [84].

**Table 5.** Advantages and disadvantages of SHF vs. SSF.

Process	Advantages	Disadvantages
SHF	Performance of enzymatic hydrolysis and fermentation under optimal conditions High production yield	Long production time based on a multistep production process
SSF	Short production time and low energy input based on a simple production process Easy to perform	Mismatch of optimal condition between enzymatic hydrolysis and fermentation Low production yield

Previous studies related to the development of SHF and SSF have suggested that the production yields are significantly lower and production costs higher when using inedible biomass as a feedstock compared with the use of synthetic medium. In other words, a major challenge to effective commercial production of isobutanol is improving the production yield.

The utilization of moderate thermophiles in the development of isobutanol-producing strains is considered an effective approach for enhancing the isobutanol production yield. Most commercially available saccharification enzymes exhibit maximum activity in the temperature range of 40–50 °C because the frequency of contact between enzyme and substrate regulated by the diffusion coefficient of the substrate increases in this temperature range, thus increasing the glucose concentration [66]. As such, there is considerable interest in the development of high-temperature SSF processes. The optimal growth temperature of *Thermoanaerobacterium saccharolyticum* is 60 °C, and an *n*-butanol-producing strain of this bacterium has been developed by genetic recombination [85]. Similarly, butanol-producing and *n*-butanol-producing strains have been developed using *T. aotearoense* [86] and *Clostridium thermocellum* [87], respectively. If bacteria such as these can be utilized as isobutanol-producing strains, the production yield could be enhanced due to the possibility of simultaneous enzymatic hydrolysis and fermentation under optimal conditions. Moreover, when a thermostable strain is used for isobutanol production, contamination by mesophiles is avoided and the need for refrigeration of fermentation tanks is eliminated, which should reduce production costs. In addition, the ability to produce isobutanol at higher temperatures might have other benefits, including expansion of production to tropical countries. Thus, SSF using moderate thermophiles enables efficient high-temperature isobutanol production from lignocellulose.

Improving the production capacity of isobutanol-producing strains based on metabolic flux analysis using substrates labeled with stable isotopes is also an effective means of increasing the isobutanol yield. Metabolic flux analysis is an experimental approach for calculating intracellular flux patterns using tracer experiments that measure the labeling pattern of metabolites of substrates labeled with stable isotopes such as  $^{13}\text{C}$  [88]. Detailed flux distributions can be obtained by combining tracer experiments, measured extracellular fluxes, and stoichiometric balances, which are useful for optimizing metabolic pathways. Indeed, it has been reported that the metabolic pathway for isobutanol production can be optimized based on  $^{13}\text{C}$  metabolic flux analysis data [89].

Most previous studies of isobutanol production by SHF and SSF used hydrothermally treated samples. Hydrothermal pretreatment is effective for lab-scale production, but it is not suitable for the pretreatment of large amounts of samples. In other words, commercial production of isobutanol must be performed on a bench scale; therefore, a pretreatment method other than hydrothermal is necessary. As the effectiveness of pretreatment varies depending on the type of feedstock and reaction conditions, additional pretreatment may be effective, such as the use of ammonia fiber expansion, dilute sulfuric acid, organosolv, or steam explosion [90].

The possibility of isobutanol production from inedible biomass depends on the substrate specificity and degradation activity of saccharification enzymes. Only inedible biomass that can be degraded by commercially available saccharification enzymes is used as feedstock. In the future, if saccharification enzymes with high degradation activity can be developed through protein engineering and applied to SHF and SSF, more inedible biomass could be used as a feedstock.

## 6. Conclusions

Global efforts are essential for reducing greenhouse gas emissions. Effective utilization of bio-based isobutanol has the potential to reduce greenhouse gas emissions, and the demand for the isobutanol is increasing in several applications, particularly for biofuels. However, practical application remains challenging due to low isobutanol yield and high production costs. To establish an economical process for isobutanol production using inedible biomass as feedstock, SHF and SSF were developed, but these processes require further improvement. Compared to SHF, the production time of SSF is reduced by simplifying the production process, and production costs may be reduced. Thus, we consider that SSF may be an economical process for isobutanol production if the productivity can be increased. To establish the process, further innovative technological developments are expected in the future.

**Author Contributions:** Conceptualization, H.A.; investigation, H.A.; writing—original draft preparation, H.A.; writing—review and editing, H.A. and A.M.; project administration, H.A.; funding acquisition, H.A. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Acknowledgments:** We are grateful to all members of the Department of Liberal Arts and Basic Science at our institute (College of Industrial Technology, Nihon University) for technical assistance and valuable discussions.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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