






Review

Clostridium sp. as Bio-Catalyst for Fuels and Chemicals Production in a Biorefinery Context

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Abstract: *Clostridium* sp. is a genus of anaerobic bacteria capable of metabolizing several substrates (monoglycerides, diglycerides, glycerol, carbon monoxide, cellulose, and more), into valuable products. Biofuels, such as ethanol and butanol, and several chemicals, such as acetone, 1,3-propanediol, and butyric acid, can be produced by these organisms through fermentation processes. Among the most well-known species, *Clostridium carboxidivorans*, *C. ragsdalei*, and *C. ljungdahlii* can be highlighted for their ability to use gaseous feedstocks (as syngas), obtained from the gasification or pyrolysis of waste material, to produce ethanol and butanol. *C. beijerinckii* is an important species for the production of isopropanol and butanol, with the advantage of using hydrolysate lignocellulosic material, which is produced in large amounts by first-generation ethanol industries. High yields of 1,3 propanediol by *C. butyricum* are reported with the use of another by-product from fuel industries, glycerol. In this context, several *Clostridium* wild species are good candidates to be used as biocatalysts in biochemical or hybrid processes. In this review, literature data showing the technical viability of these processes are presented, evidencing the opportunity to investigate them in a biorefinery context.

Keywords: *Clostridium* sp.; fuels; ethanol; butanol; acetone; 1,3-propanediol; biorefinery; biomass

1. Introduction

In 2018, Global Footprint Network stated that natural resources were being used 1.7 times faster than the Earth's capacity for regeneration [1]. Water, soil, and air pollution generated by anthropogenic actions have caused climate change, resource depletion, global warming, water crises, and the increase of natural disasters in recent years [2]. Many researchers and initiatives are committed to search and develop new techniques, feedstocks, and processes towards a sustainable economy that will be less dependent on oil as a source of energy and chemical production [3]. One approach is to progressively change our primary source of fuels and chemicals, decreasing the dependency on oil and oil products due to oil's environmental impact, depletion possibility, and market price oscillations caused by oil and financial crises. The Sustainable Development Goals adopted by global leaders at the 2015 United Nations Summit set the path for 17 goals to be achieved by 2030, such as: affordable and clean energy (SDG7); industries, innovation and infrastructure (SDG9); responsible production and consumption (SDG12); and climate action (SDG13) [4].

Many feedstocks can be used to produce renewable and sustainable fuels and chemicals, especially residual organic materials, industrial waste-streams, and by-products. The use of glycerol, molasses,

corn steep liquor, energy crops, grass silage, cattle slurry, harvest residues, and food processing waste can reduce operational costs (associated with feedstock acquisition) and environmental impact due to improper disposal of residue [3,5–10]. These are renewable feedstocks that can be transformed into valuable biofuels (e.g., biobutanol and bioethanol) and biochemicals (lactic acid, butyric acid, and succinic acid), achieving a balance between environmental sustainability and economic growth in a process named biorefining [10]. Biorefining is defined as “the sustainable processing of biomass into a spectrum of marketable food and feed ingredients, bio-based products (chemicals, materials) and bioenergy (biofuels, power and/or heat)” [11]. In a biorefinery, components of biomass are organized as building blocks (e.g., cellulose, hemicellulose, lignin, and proteins) and the biorefinery concept encompasses the whole processing of biomass, considering upstream, midstream, and downstream processing [3,12]. The main challenge is the feasible production of biofuels and biochemicals in a cost effective, efficient, and sustainable way [3]. Although using renewable feedstock, biorefinery sustainability should also consider others aspects, such as land use for biomass production, soil, and air quality during processing, and environmental and economic impacts [13].

Considering biochemical conversion processes, microbial cells are a central aspect of biomass conversion, functioning as biocatalysts that convert liquid, solid, or gaseous substrates into alcohols, acids, enzymes, and other products through a series of metabolic reactions. In particular, *Clostridium* species have been involved in many important fermentative processes, such as the production of butanol, acetone, ethanol, acetic acid, lactic acid, succinic acid, 1,3-propanediol, and more [14–17]. Therefore, the current study reviews fundamental aspects of important *Clostridium* species and their biochemical pathways to highlight major theoretical concepts that are present in many processes currently discussed in literature. This work also highlights important biomolecules and feedstocks that have a great potential in the biorefinery concept and would play an important role towards a more sustainable bioeconomy.

2. *Clostridium* sp.

Prazmowski proposed the genus *Clostridium* through the type species *Clostridium butyricum* in 1880. This genus then became the general category for anaerobic, spore forming and Gram-positive microorganisms. There are about 228 species and subspecies currently known, some of which highly heterogeneous, that present several phenotypes [18]. Among the species of the genus there are those that synthesize quinones and cytochromes, acidophiles, thermophiles, and psychrophiles [19]. According to Lawson and Rainey there are phylogenetic and phenotypic incoherencies in the genus *Clostridium*. The researchers defend the restriction of the genus *Clostridium* to *Clostridium* cluster I as *Clostridium sensu stricto* [18].

2.1. General Aspects

Generally, bacteria of the *Clostridium* genus are rod-shaped with Gram-positive staining. Single cell diameters may vary from 0.3 to 2.0 μm and lengths from 1.5 to 20 μm ; they are usually arranged in pairs or in short chains with rounded or pointed ends. With the presence or absence of flagella, a single cell may be motile, usually with peritrichous flagella, or immotile. They do not reduce sulfates and are, generally, catalase negative [18]. Most species have sporulation capacity, which can be directly affected by presence of oxygen, because they are anaerobic. However, species of *Clostridium* genus have different oxygen tolerance capacities [19]. Most species are chemotrophic; some are chemoautotrophic or chemolithotrophic. They may be saccharolytic, proteolytic, none, or both. Metabolically they are very diverse, with optimum temperature between 10 and 65 $^{\circ}\text{C}$. They are distributed in the environment. Many species produce potent exotoxins, and some species are pathogenic to animals, both by infection of wounds and by absorption of toxins [20].

When exposed to nutrient-depleting conditions, alternative metabolic pathways of clostridia are activated. Sporulation starts at the beginning of the stationary phase as an adaptation response and can affect end products related to energy metabolism, since it involves several metabolic pathways

associated to biochemical, morphological, and physiological changes. Although the lack of nutrients commonly triggers sporulation, a source of energy is still needed to fuel macromolecular synthesis. Complete depletion of all or even a single nutrient can lead to cell death [21].

2.2. Workhorse Species

Many *Clostridium* species produce a wide variety of organic acids and solvents of industrial interest, such as ethanol, butanol, 1,3-propanediol, and acetone, among others. The most important species for a biorefinery context will be discussed in this review. Table 1 shows the most important species and their main products.

Table 1. Main products and production data for *Clostridium* species of interest to biorefinery.

Species	Main Products	P _{Max} ¹ (g/L)	Q _{Max} ¹ (g/L.h)	Substrate or Feedstock	V ¹ (L)	Time (h) ¹	Ref. ¹
<i>C. carboxidivorans</i>	Ethanol	5.55		100% CO	1.2	500	[22]
	Butyric acid	1.43	NA ²				
	Butanol	2.66					
	Ethanol	23.93	0.143	20% CO, 5% H ₂ , 15% CO ₂ , 60% N ₂	8	360	[23]
<i>C. ragsdalei</i>	Ethanol	13.2 ³	NA	40% CO, 30% H ₂ , 30% CO ₂	2.4	290	[24]
		19	NA	38% CO, 28.5% H ₂ , 28.5% CO ₂ , 5% N ₂	2.4	470	[25]
<i>C. ljungdahlii</i>	Ethanol	20.7 ⁴	0.374	60% CO, 35% H ₂ , 5% CO ₂	4	2014	[26]
		48	NA	55% CO, 20% H ₂ , 10% CO ₂ , 15% N ₂	1	560	[27]
<i>C. kluyveri</i>	n-caproic acid	8.42	0.07	Ethanol and acetate (10:1)	0.1	120	[28]
<i>C. beijerinckii</i>	Butanol	34.77 ⁴	0.48	Corn stover hydrolysate	1.25	72	[29]
		11.92	0.17	Corn cob hydrolysate	50	72	[30]
		11.77	0.10	Sugarcane juice	1.5	100	[31]
	Isopropanol	3.41	NA	Glucose	0.06	48	[32]
<i>C. saccharobutylicum</i>	Butanol	12.8	0.257	Corn stover hydrolysate	5	48	[33]
	ABE 5	19.9	-				
<i>C. saccharoperbutylacetonicum</i>	Butanol	64.6 ⁶	0.673	Glucose	0.5	96	[34]
	Acetone	30.6	0.319				
	ABE	97.3	1.01				
<i>C. thermocellum</i>	Ethanol	13.66	NA	Cellulose	0.02	120	[35]
<i>C. phytofermentans</i>	Ethanol	7.0	0.03	AFEX™ -treated corn stover ⁷	0.3	250	[36]
<i>C. acetobutylicum</i>	Butanol	44.6 ⁸	2.13	Glucose	4	88	[37]
	Ethanol	33 ⁹	0.47	Glucose	0.2	185	[38]
	Acetone	7.59 ¹⁰	0.13	Glucose	5	54	[39]
<i>C. butyricum</i>	1,3-propanediol	93.7	3.30	Pure glycerol	1	32	[40]
<i>C. cellulolyticum</i>	Ethanol	2.5 ¹⁰	0.007	Crystalline AVICEL ¹¹ Cellulose	0.05	336	[41]

Table 1. Cont.

Species	Main Products	P _{Max} ¹ (g/L)	Q _{Max} ¹ (g/L.h)	Substrate or Feedstock	V ¹ (L)	Time (h) ¹	Ref. ¹
<i>C. cellulovorans</i>	Butanol	3.37	0.046	Corn cob	2	72	[42]
<i>C. pasteurianum</i>	Butanol	17.8	7.8 ¹²	Pure glycerol	0.09	48	[43]
	1,3-propanediol	9.5	0.2		0.09	48	
<i>C. perfringens</i>	1,3-propanediol	40	2.0	Pure glycerol	3	48	[44]

¹ P_{Max}: maximum production reported; Q_{Max}: maximum productivity for the maximum production reported; V: production volume; time: fermentation time; Ref: reference; ² NA: not available data in reference; ³ using PLBC (Poultry litter biochar) medium with MES (4-morpholineethanesulfonic acid); ⁴ this production was achieved with integrated product recovery method; ⁵ ABE: sum of acetone, butanol, and ethanol concentrations; ⁶ butanol, acetone, and ABE production with immobilized cells; ⁷ ammonia fiber expansion (AFEX™) is an alkaline pretreatment using ammonia as a catalyst; ⁸ this production was achieved with a continuous process with ex situ butanol recovery and a mutant strain obtained by chemical mutagenesis; ⁹ mutant strain; ¹⁰ genetic engineered strain; ¹¹ AVICEL: is a purified, partially depolymerized alphacellulose made by acid hydrolysis of specialty wood pulp; ¹² productivity obtained in 1 L fermenter with 0.4 L of medium for 710 h, with cell recycle.

2.2.1. *Clostridium carboxidivorans*

C. carboxidivorans was first discovered in the sediment of an agricultural decantation pond at the State University of Oklahoma, USA, after enriching the environment with carbon monoxide (CO), as a strategy to identify bacteria species capable of assimilating CO as a substrate [14].

It is an aerotolerant Gram-positive anaerobic bacterium with rod-shape cells that may occur in pairs or isolated. It has motility and a rare sporulation mechanism. The optimum temperature for growth is 38 °C. This species presents autotrophic growth when in the presence of carbon dioxide (CO₂) and CO, but is also able to grow with several organic sources, such as fructose, galactose, glucose, and mannose [45]. It produces acetic acid, ethanol, butyrate, and butanol as the final metabolic products [14,45]. From syngas (mixture of mainly CO, CO₂, and H₂), almost 24 g/L of ethanol production have been reported [23] (Table 1). The surface of *C. carboxidivorans* cells are hydrophilic but also capable of being attracted by hydrophobic molecules, liquids, and surfaces, and capable of interacting with them when immersed in water [46].

In the genomic analysis of *C. carboxidivorans*, to understand biofuel production pathways genetic determinants were observed for CO use and production of acetate, ethanol, and butanol. One example is the presence of a gene encoding a dehydrogenase characteristic of the ABE (acetone-butanol-ethanol) fermentation, previously described for *C. acetobutylicum* and *C. beijerincki*, responsible for converting acetyl-CoA to acetaldehyde and ethanol, and acetyl-CoA to butyryl-CoA and butanol via butyraldehyde. However, genes present in other *Clostridium* strains that encode proteins for acetone production were not found in *C. carboxidivorans* [47].

2.2.2. *Clostridium ragsdalei*

C. ragsdalei (ATCC PTA-7826) “P11” was first isolated from duck pond sediment, in a laboratory in Norman, OK, USA. This species transforms waste gases (e.g., syngas and refinery wastes) into useful products [48]. It became of the major species capable of fermenting synthesis gas. *C. ragsdalei* uses the Wood–Ljungdahl pathway to assimilate CO, CO₂, and H₂ to produce acetate [49–51]. It is poorly inhibited by acetic acid compared to other syngas-fermenting strains, such as *Clostridium coskatii* [52]. This microorganism has been studied for acid and solvent production from synthesis gas. Some residues, such as corn steep liquor (CSL), have been used as a source of nutrients during fermentation in order to reduce production costs. The growth of *C. ragsdalei* in CSL depends on trace metals, NH₄, and reducing agents, due to its low resistance to oxygen [51].

Among the main products of its metabolism is ethanol, with the highest concentration of 19 g/L reported after 470 h of syngas fermentation [25] (Table 1). The conversion of the acetate produced in the acidogenic phase into ethanol in the solventogenic phase depends on the pH of the medium, because solventogenesis mainly begins when the PH value is reduced [53].

2.2.3. *Clostridium ljungdahli*

C. ljungdahli ATCC 49,587 was isolated from chicken yard waste [54]. Rod-shaped cells with motility and rare formation of spores are some of the physical characteristics of this species. The pH and temperature range for cell growth are 4.0–7.0 and 30–40 °C, respectively. However, optimum growth conditions are 37 °C and pH 6.0.

Autotrophic or chemoorganotrophic growth are observed for this species, with H₂-CO₂ or CO and formate, ethanol, pyruvate, fumarate, erythrose, threose, arabinose, xylose, glucose, and fructose as carbon sources. It is incapable of assimilating methanol, ferulate, trimethoxybenzoate, lactate, glycerol, citrate, succinate, galactose, mannose, sorbitol, sucrose, lactose, maltose, or starch. The major metabolic end product is acetic acid in the acidogenic phase, with trace amounts of ethanol in the solventogenic phase [19,54]. However, some studies have reported 20 g/L [26] and 48 g/L [27] of ethanol during syngas fermentation using this species (Table 1).

2.2.4. *Clostridium kluyveri*

C. kluyveri is distinguished by both its nutritional requirements and its ability to ferment ethanol to caproate [28]. Cells are motile, with peritrichous flagella, and generally occur singly, and occasionally in pairs or chains. They are weakly Gram-positive, but quickly become colored with Gram-negative staining. The endospores are oval, terminal, or subterminal and swell the cell. Ultra-structural studies demonstrated a five-layer cell wall and a three-layer plasma membrane. Growth is slow and occurs between 19 and 37 °C, with optimum temperature of 35 °C.

The strains require ethanol, CO₂, or sodium carbonate, and a high concentration of yeast autolysate or acetate, propionate, or butyrate for growth. In the presence of CO₂ or carbonate and acetate or propionate, ethanol is converted into butyrate, caproate, and H₂. H₂ is formed and small amounts of butyrate can be detected [55].

2.2.5. *Clostridium beijerinckii*

C. beijerinckii cells present a straight rod morphology and rounded ends. They are motile with peritrichous flagella, occurring singly, in pairs, or in short chains. They are Gram-positive, becoming Gram-negative in the older cultures. The endospores are oval, eccentric to the subterminal, and swell to the cell, without exosporium or appendages. The ideal growth temperature is 37 °C, but they also grow well at 30 °C. The growth is stimulated by a fermentable carbohydrate and is inhibited by 6.5% NaCl or 20% bile. Yeast extract supplies nutritional requirements of the cells [19,56].

C. beijerinckii is one of the major isopropanol producing bacteria with several known strains (ATCC 25752, CIP 104308, DSM 791, JCM 1390, LMG 5716, NCTC 13035) [38]. It is also known as a butanol producer [30,31]. Aiming at biorefinery interest, this species uses lignocellulosic hydrolysate materials and produces butanol and acetone or isopropanol. High butanol production from sugarcane juice [30] or corn cob hydrolysate [31] has been reported (Table 1). This species produces alcohol dehydrogenase but only some strains specifically produce isopropanol dehydrogenase, responsible for the conversion of acetone to isopropanol via isopropanol-butanol-ethanol (IBE) fermentation [19,32]. It has been reported that Zn²⁺, Ca²⁺, and Fe²⁺ improve butanol and isopropanol production [32]. They also increase the specific activity of hydrogenase, stimulating H₂ production [57].

RNA transcription analysis has shown that the metabolic pathway used by *C. beijerinckii* to produce 1,2-propanediol, propionate, and propanol is similar to the pathway used by *C. phytofermentans*. When L-rhamnose is used as a carbon source, a bacterial microcompartment (BMC) is formed, which is directly involved in propanol and propionate formation. However, in the presence of glucose there is no formation of BMC and, consequently, propanol, and propionate are not produced [56,58].

2.2.6. *Clostridium saccharobutylicum*

Morphologically *C. saccharobutylicum* cells are characterized by a rod shape with rounded ends of 3.8–10 µm in length and occur individually, in pairs, or short chains. Their cells are motile with peritrichous flagella. A Gram-positive cell wall is characteristic of this species, but older cultures stain with Gram-negative. The spores have an oval shape, with a terminal location inside the cell. The ideal temperature and pH ranges for growth and solvent production are 30–34 °C and pH 6.2–7.0, respectively [19].

C. saccharobutylicum produces acetone, butanol, ethanol, CO₂, H₂, and acetic and butyric acids as fermentation products [19]. It is mainly used to ferment lignocellulosic hydrolyzed materials, such as corn stover, to produce butanol in ABE (acetone-butanol-ethanol) fermentation (Table 1) [33,59].

2.2.7. *Clostridium saccharoperbutylacetonicum*

Morphologically, *C. saccharoperbutylacetonicum* cells are straight rods, which are found individually or in pairs. Cells are mobile with peritrichous flagella. They stain Gram-positive as young cells, whereas Gram-negative cells are observed in older cultures. They form oval spores and grow well in temperatures between 25 and 35 °C and a pH range of 5.6–6.7 [19].

C. saccharoperbutylacetonicum produces acetone, butanol, ethanol, CO₂, H₂, and acetic and butyric acid as final fermentation products [19,56]. It is considered outstanding as a model species for industrial application of acetone-butanol-ethanol (ABE) fermentation, producing 73%–85% of butanol and sporulates with low frequency [56]. Extractive fermentation with *C. saccharoperbutylacetonicum* immobilized cells resulted in high total butanol concentration (64.6 g/L) (Table 1) [34].

The use of genetic engineering techniques contributes to a better understanding of genetic information. Therefore, the strain *C. saccharoperbutylacetonicum* N1-4C demonstrated better plasmid stability and a slight increase in ABE production, probably due to the improvement in the efficiency of acids produced assimilation during the acidogenic phase [60]. The silencing of the *pta* and the *buk* genes, responsible for the expression of phosphotransacetylase and butyrate kinase, respectively, did not eliminate the formation of acetyl or butyrate. This indicated that *C. saccharoperbutylacetonicum* has additional routes for acid production [61].

2.2.8. *Clostridium thermocellum*

C. thermocellum is a thermophilic bacterium that grows between 60 and 64 °C. The culture medium influences the motility of this species. They are Gram-negative but possess a Gram-positive type cell wall without lipopolysaccharide. They are morphologically characterized by being straight or slightly curved cells, which can be found individually or in pairs. The spores are oval and terminal, and swell the cell [62].

C. thermocellum is capable of converting cellulosic waste into ethanol and H₂ [62]. It can also produce lactate and acetate as main products [63]. Growth in most media requires the presence of cellulose, cellobiose, or hemicelluloses [62]. A genetically modified strain produced 5 g/L of ethanol from microcrystalline cellulose [63] and, using a statistical approach, Balusu et al. [35] reported production of more than 13 g/L of ethanol from cellulosic biomass with a wild-type strain (Table 1).

A thermodynamic analysis of metabolomic data of *C. thermocellum* showed that bottlenecks of ethanol production from cellobiose are distributed across five reactions under high ethanol levels and identified best intervention strategies for ethanol production [64].

2.2.9. *Clostridium phytofermentans*

C. phytofermentans is morphologically characterized as straight sticks that measure 0.5–0.8 × 3–15 µm, able to occur alone or in pairs. They are stained Gram-negative despite having a Gram-positive wall and its motility is provided by one or two flagella. Sporulation for this species is terminal with

round spores. It is an obligate anaerobic bacterium with wide temperature and pH ranges for growth: from 15 to 42 °C and pH of 6.0 to 9.0. Optimum temperature and pH are 37 °C and 8.0, respectively [65].

C. phytofermentans uses a wide variety of plant polysaccharides as a substrate, such as cellulose. It produces ethanol, acetate, CO₂, and H₂ as the main products [65]. Due to its capacity to convert cellulosic biomass to ethanol, *C. phytofermentans* seems promising in the biorefinery context, using lignocellulosic material as a source of substrate, with references reporting 7 g/L of ethanol from corn stover (Table 1) [36]. The use of algae biomass from sewage without pre-treatment by *C. phytofermentans* yielded 0.19 g of ethanol per g of biomass using 2% w/v of algae [66].

2.2.10. *Clostridium acetobutylicum*

A strictly anaerobic, Gram-positive and spore-forming bacterium, *C. acetobutylicum* was first isolated by Chaim Weizmann in 1916. Weizmann discovered that this species produces ethanol and butanol from starch. This isolated strain was used in the production of acetone during World War I as a precursor to cordite production [67]. The *C. acetobutylicum* strain responsible for ABE fermentation in the ratio 7:2:1 was only isolated in 1980 [68].

C. acetobutylicum has a high amylase activity, being able to metabolize a variety of carbohydrates, such as starch, glucose, and sucrose [69,70]. Recent studies point out the potential use of *C. acetobutylicum* for the conversion of algal hydrolysates, algae biomass, and galactose into value-added bioproducts [71]. However, *C. acetobutylicum* is less effective for complex carbon sources, i.e., lignocellulosic hydrolysates, which may contain toxic substances derived from the dehydration of sugars and aromatics from lignin that are generated during biomass pretreatment [70].

From the carbohydrate sources available in the culture medium, *C. acetobutylicum* synthesizes acetic acid and butyric acid, which are subsequently converted into organic solvents, such as acetone, butanol, and ethanol [21]. Among the known microorganisms capable of producing butanol, *C. acetobutylicum* is the one with the highest production and, therefore, one of the most commonly used species for butanol production [68]. In a continuous process with ex situ butanol recovery and a mutant strain obtained by chemical mutagenesis, 44.6 g/L of butanol was achieved (Table 1) [37].

2.2.11. *Clostridium butyricum*

An anaerobic and saccharolytic bacterium, fermentation of *C. butyricum* is similar to that of several species of *Clostridium*. *C. butyricum* is found in the intestinal tract of humans and animals, and this bacterium can be used as an animal feed additive because of its probiotic properties. Butyric acid, one of the short chain fatty acids produced in its fermentation, has anti-inflammatory effects and aids in the proliferation of intestinal mucosal cells [72]. *C. butyricum* is also frequently found in soil converting organic matter and helping soil mineralization, having a relevant role in ecology [73].

The metabolic products of *C. butyricum* are short chain fatty acids, acetic acid, butyric acid, and propionic acid. H₂, butanol, and 1,3-propanediol can also be obtained through fermentation of sugar and glycerol [72]. *C. butyricum* is considered one of the most important 1,3-propanediol producers. This is due to the high conversion efficiency, the soft cultivation system, and the non-sterile process that contribute to competitive and cheaper production [74]. More than 90 g/L of 1,3-propanediol was produced by this species with pure glycerol as the carbon source [40].

2.2.12. *Clostridium cellulolyticum*

C. cellulolyticum cells are mesophilic anaerobic bacteria capable of growing on crystalline cellulose [75,76]. Its name originates from the Greek words cellulose (cellulose) and lyticus (dissolution). They are rod-shaped with peritrichous flagella [76]. *C. cellulolyticum* is referred to as mesophilic cellulolytic clostridium, which secretes cellulosome, a multi-enzyme complex capable of converting cellulose into metabolites of interest, such as ethanol, acetate, lactate, and H₂. The cellulosome converts cellulose to cellobiose and cellobiose, being then incorporated and metabolized,

generating other metabolites [75,76]. A genetically engineered strain was able to produce 8.5 times more ethanol from crystalline cellulose than wild-type cells (Table 1) [41].

2.2.13. *Clostridium cellulovorans*

C. cellulovorans is a cellulolytic mesophilic anaerobic bacterium capable of degrading pectin and hemicellulose, in addition to cellulose [77]. It had its genome sequenced in 2010 after being isolated from wood chips [78]. Its main product is butyric acid and among the by-products are acetic acid, lactic acid, formic acid, ethanol, H₂, and CO₂. It degrades cellulose efficiently due to the expressive number of cellulosomal genes. It is capable of using a wide range of carbon sources besides cellulose, such as lactose, glucose, galactose, maltose, sucrose, cellobiose, pectin, and xylan [78].

Due to the variety of carbon sources and the fact that it produces butyric acid, *C. cellulovorans* is a cellulolytic organism with great potential to produce butanol, requiring only one more stage of enzymatic activity [78]. The fermentation process engineering of *C. cellulovorans* enabled a high butanol production directly from corn cob (Table 1) [42].

2.2.14. *Clostridium pasteurianum*

An acidogenic saccharolytic, *Clostridium* is capable of fixing molecular nitrogen, dispensing other nitrogen sources [67]. It presents Gram-positive cells only in very young cultures. It has robust growth even in simple media and in non-sterile conditions.

It is capable of producing chemicals, such as 1,3-propanediol and n-butanol, from renewable raw materials, such as glycerol derived from biodiesel and glucose from hydrolysis of biomass, respectively [71,79]. Ethanol, acetic acid, butyric acid, lactic acid, H₂, and CO₂ are also among the products of its fermentation. Although necessary to ensure intracellular redox balance, the organic acid production pathway competes with that of the products of interest. There is also selectivity in the proportion of the products generated, between 1,3-propanediol and n-butanol, for example, which is strongly influenced by factors such as nutrient composition and growing conditions. Other factors that may influence this ratio are culture pH, inoculum condition, and medium supplementation with yeast extract, ammonia, organic acids, phosphate, and iron [79]. An increase in the production of butanol by *C. pasteurianum* DSM 525 was observed when lactic acid was used with glycerol. Lactic acid can buffer the medium, stabilizing the pH between 5.7 and 6.1 [80].

2.2.15. *Clostridium perfringens*

C. perfringens was first isolated by Welch and Nuttall in 1892 from a human corpse in an advanced state of decomposition [20]. It is widely distributed in soils and the food tracts of almost all warm-blooded animals. Generally, it is found to be an invader of the food tract after death. Caution is required in the isolation of this *Clostridium* after death. This agent is found in the gaseous gangrene of man and animals, or associated with other anaerobes, in this process [20]. *C. perfringens* is a straight and thick rod, usually individual, in pairs, or, rarely, in strings. It produces oval and small spores, which are not formed in very acidic medium. It is classified as a Gram-positive bacteria, but becomes Gram variable with subcultures [20].

It produces acid and gas from glucose, levulose, galactose, mannose, maltose, lactose, sucrose, xylose, trehalose, raffinose, starch, glycogen, and inositol. Fermentation products include acetic acid, butyric acid, and 1,3-propanediol [20]. In fed-batch fermentation, an isolated new strain (*C. perfringens* GYL) showed a maximum productivity of 2.0 g/(L·h), and produced 40.0 g/L 1,3-propanediol, with a high yield from glycerol (Table 1) [44].

2.2.16. *Clostridium tyrobutyricum*

Anaerobic and spore-forming, *C. tyrobutyricum* [81] is widely used in the production of butyric acid due to its high production in simple media [82]. It is also the biggest cause of problems in the dairy industry thanks to this ability of converting lactate to butyrate in the presence of acetate. During

fermentation, lactate is the electron donor and the acetate is the electron acceptor, generating butyrate. Growth of saccharolytic clostridia is observed in most environments where the oxygen tension is suitably low [81,82].

2.2.17. Other Species

Clostridium drakei was initially described as *C. scalatogenes*, but 16S RNA analysis determined that it was a new species which was named after a researcher, Drake, in recognition of his contributions to the physiology and ecology of acetogens. Spores are located in the terminal portion of the cell. They are mandatory anaerobes, with an optimal growth temperature of 30 to 37 °C and optimal pH of 5.5–7.5. *C. drakei* grows autotrophically with H₂/CO₂ or CO, and also chemiotrophically. The main end products are acetic acid, ethanol, butyrate, and butanol [14].

Clostridium difficile was initially isolated from newborn feces and referred to as *Bacillus difficilis*. The name refers to the difficulty found in isolating and studying this species. *C. difficile* produces isocaproic and valeric acid. The cells are shaped like rods that join in aligned groups by the tips of two to six cells. They have motility and the presence of peritrichous. The sporulation capacity is observed when induced by culturing in specific medium, and the growth temperature ranges from 25 to 45 °C. Acetic, butyric, lactic acid, and butyrate are the main products obtained by this species [19].

Clostridium autoethanogenum was isolated from rabbit feces. It is strictly anaerobic, with motility and forms sporulation. It is able to produce ethanol, acetate, and CO₂ from CO as a carbon source. The optimal pH and temperature for growth is 5.8–6.0 and 37 °C, respectively [83]. When cultured with H₂, the assimilation of CO by *C. autoethanogenum* increases and the metabolic pathway to ethanol production is stimulated, reducing CO₂ generation [84].

2.3. Fermentation and Biochemical Pathways

Organic acids and alcohols can be obtained by anaerobic fermentation performed by *Clostridium* from organic or inorganic carbon sources. The fermentation processes of the different species are divided into three main fermentations: ABE (acetone-butanol-ethanol), IBE (isopropanol-butanol-ethanol), and HBE (hexanol-butanol-ethanol). The latter was recently proposed by a research group that has been studying the *C. carboxidivorans* P7 strain [85,86].

Independently of the end products, each strain can use different metabolic pathways based on the available carbon source and its genetic information. Different carbohydrates, for example, are fermented through different pathways by clostridia. In the case of hexoses, their metabolism follows the Embden–Meyerhof–Parnas (EMP) pathway, whereas the metabolism of pentoses takes place through the pentose phosphate (PP) pathway [86]. On the other hand, to assimilate CO, CO₂, and H₂ for HBE fermentation, *C. carboxidivorans* uses the Wood–Ljungdahl pathway [85,86]. These fermentation processes and biochemical pathways will be discussed in this section.

2.3.1. Acetone-Butanol-Ethanol (ABE) Fermentation

ABE fermentation was used on a large scale during the First World War. Acetone was produced by *C. acetobutylicum* to be used by England for warlike applications. However, the development of the petrochemical industry after the Second World War, decreased the interest in ABE fermentation to obtain those products. Recently, environmental problems related to the use of fossil fuels and economic reasons have again increased the interest for ABE fermentation [87,88].

ABE fermentation is composed of two phases: acidogenic and solventogenic. The first is characterized by organic acid production (butyric and acetic), and the second by assimilation of these acids as a carbon source for subsequent transformation into acetone, butanol, and ethanol [56,87]. Generally, molar the acetone:butanol:ethanol ratio is 6:3:1, but it can vary from species to species [56,87]. The most common and best known solventogenic clostridial strains for commercial butanol fermentation are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [86].

The use of lignocellulosic biomass in ABE fermentation for butanol production has been studied by several authors as a solution for the environmental issues mentioned. However, carbon catabolic repression (CCR) reduces the efficiency of the process since glucose, usually produced after a pre-treatment of the biomass, suppresses the use of other sugars, such as xylose and arabinose, which are also produced [89,90]. For butanol production by *C. acetoperbutylaceticum*, Noguchi et al. [89] used a different pre-treatment and saccharification of lignocellulosic material that generates cellobiose rather than glucose, and xylose, avoiding CCR. Butanol productivity increased with this strategy. Recently, it was also demonstrated that biochar (a by-product obtained from thermochemical conversion of biomass under oxygen limited conditions) can serve as a cost-effective substitute for mineral and buffer solutions in ABE fermentation [91].

2.3.2. Isopropanol-Butanol-Ethanol (IBE) Fermentation

Butanol has been considered an advanced biofuel and its production at a scale of billions of liters by ABE fermentation may result in oversupply of acetone [92]. IBE fermentation is a solution to the acetone problem, because in this process naturally-occurring strains can convert acetone in isopropanol, resulting in the production of isopropanol, butanol, and ethanol. Additional advantages are the possibility to use this mixture directly as a fuel and the removal of a high corrosive substance with low fuel properties [93].

C. beijerinckii is one of the main species that produce IBE. It has been identified that the BGS1 strain has 16 coding sequences related to alcohol dehydrogenase, including the *sAdhE* gene that encodes isopropanol dehydrogenase, responsible for acetone to isopropanol conversion. However, not all *C. beijerinckii* strains produce isopropanol dehydrogenase [32]. Another inconvenience, as depicted by Vieira et al. [92], is that natural IBE producers are more sensitive to product inhibition (caused mainly by butanol) and, consequently, are less efficient than ABE producers.

2.3.3. Hexanol-Butanol-Ethanol (HBE) Fermentation

C. carboxidivorans produces hexanol and hexanoic acid from synthesis gas (mainly CO, H₂, and CO₂) [94]. As this bacterium also produces butanol and ethanol, the term HBE (hexanol-butanol-ethanol) fermentation was introduced by Fernandez-Naveira et al. [86]. HBE fermentation is a term derived from ABE fermentation and known for solventogenic bacteria [94], as already mentioned.

C. carboxidivorans is able to start the solventogenic phase without a drastic pH reduction, which is the case of ABE fermentation. Thus, buffering the culture medium for HBE fermentation by *C. carboxidivorans* may induce higher acid production and promote greater conversion in the solventogenic phase. However, further investigation and applications of genetic engineering are necessary to reduce the inhibitory effect of high concentrations of solvents and acids produced [86].

2.3.4. Embden-Meyerhof-Parnas (EMP) Pathway and Pentose Phosphate (PP) Pathway

EMP and PP pathways for *Clostridium* species to metabolize hexoses and pentoses, respectively, have been shown by several authors [86,95,96]. Agro-industrial wastes, commonly used as feedstocks for biofuel production, generate mainly glucose, arabinose, mannose, xylose, fructose, sucrose, and lactose as substrates after a pre-treatment process [86].

The nature of the sugars is a key parameter that will affect the efficiency of the fermentation process and the production of metabolites [86]. Monosaccharides are transported by a faster system than disaccharides. In the case of glucose and fructose, EMP is directly used. For galactose, for example, after being phosphorylated, galactose-6-P is metabolized via the tagatose 6-P pathway and subsequently enters the glycolytic pathway. These differences result in different consumption rates between sucrose and lactose [86,97]. In the case of pentoses, which are usually mixed with hexoses, there is the carbon catabolic repression (CCR) issue, which suppresses pentose consumption because of the preferable use of glucose [89,90].

2.3.5. Wood–Ljungdahl Pathway

The Wood–Ljungdahl (WL) pathway provides acetate from inorganic sources, such as H₂, CO, and CO₂. Acetogenic bacteria follow the WL pathway to produce biofuels from CO, CO₂, and H₂, or syngas/waste gas [86].

The WL pathway comprises two branches: methyl and carbonyl branches both contribute to acetyl-CoA formation and start with the assimilation of CO or CO₂ [98]. In the methyl branch, formate is obtained through the reduction of CO₂. In the carbonyl branch, CO or CO₂ can be taken directly and converted into acetyl-CoA [86]. Growth of acetogenic bacteria is possible because of the formation of acetate from 2 mols of CO₂, with H₂ as a reducing agent and, therefore, this pathway must be coupled with ATP formation. This is believed to be the oldest biochemical pathway, responsible for producing biomass and ATP in the ancient world [85]. In the first step of the methyl group, the possibility of CO assimilation followed by the action of CO deshydrogenase generates two protons and two electrons that can be used as inputs to format deshydrogenase action on the following reaction. In other words, the WL pathway is capable of sustaining the autotrophic growth of the microorganism only with CO, even without H₂ for electron donation [68,71]. In heterotrophic conditions, alcohols, organic acids, and simple sugars are able to donate electrons to the WL pathway [72].

In the Wood–Ljungdahl pathway, acetyl-CoA is a common intermediate of both branches. The acetyl-CoA formed by the WL pathway can be used for direct biomass formation or to undergo action of the phosphotransacetylase (PTA) followed by the acetate kinase (ACK), generating 1 ATP and forming 1 acetate. Alternatively, acetyl-CoA can also be converted to acetaldehyde, then to ethanol or butyryl-CoA and, subsequently, into butyrate and/or butanol, or into hexanoyl-CoA and then hexanoate and/or hexanol [86].

Oxygen inhibits various enzymes from the WL pathway and its presence may increase the redox potential. Therefore, it is considered one of the most toxic gases during the syngas fermentation [72]. However, some acetogenic microorganisms are tolerant of O₂ concentrations ranging from 0.5% to 6% of saturation [73].

3. Feedstock for Anaerobic Processes

3.1. Glycerol

Known for thousands of years through soap production, the glycerol molecule is considered the oldest isolated by man [99]. Its name comes from the Greek word “glykys”, which means sweet [98]. It is an organic molecule of alcohol function that presents three hydroxyl groups in its structure, which gives it the characteristics of solubility in water [99]. It has no known toxic effect on either man or the environment [100,101]. It is a viscous liquid, with no smell, no color, a sweet taste and a high boiling point [100,101]. It is stable under several conditions [99]. There are about 1500 known applications for glycerol, which include those of the pharmaceutical, food, and cosmetics industries [102]. It is commercialized in the form of glycerin, a mixture of glycerol and impurities from the production process with purity varying between 95% and 99% [100,101]. It is also found in the form of “crude glycerol”, originating mainly from the biodiesel industry, with 60%–80% glycerol in its composition [100–102].

Equivalent to 10% (*w/w*) of biodiesel production [103], glycerol supply no longer depends on demand. Glycerol is in excess supply in the market due to the large growth of the biodiesel industry since 2006 [101], making it a low-cost raw material. The price reduction of glycerol enables its use in new productive processes of value-added chemicals and its entry into countries where it was not previously accessible [99].

In addition to being used in chemical processes for the generation of several products, glycerol can also be employed as a substrate in biotechnological processes. Some examples of products generated by glycerol metabolization by *Clostridium* species are: 1,3-propanediol (*C. butyricum*, *C. butylicum*, *C. pasteurianum*, *C. perfringens*, *C. beijerinckii*, *C. kainantoi*), acetic acid (*C. butyricum*, *C. butylicum*, *C. pasteurianum*, *C. perfringens*, *C. beijerinckii*, *C. carboxidivorans*), butyric acid (*C. butyricum*, *C. pasteurianum*,

C. carboxidivorans), lactic acid (*C. pasteurianum*), butanol (*C. pasteurianum*, *C. acetobutylicum*, *C. beijerinckii*, *C. butylicum*, *C. carboxidivorans*), ethanol (*C. butyricum*, *C. pasteurianum*, *C. perfringens*, *C. acetobutylicum*, *C. beijerinckii*, *C. butylicum*, *C. carboxidivorans*), acetone (*C. acetobutylicum*, *C. beijerinckii*, *C. butylicum*), propionic acid (*C. butylicum*), propanol (*C. butylicum*), 1,2-propanediol (*C. butylicum*), and H₂ (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii*) [14,44,57,104–106]. The elemental composition of crude glycerol from biodiesel production is around 53% carbon, 36% oxygen, and 11% nitrogen, which makes it a good source of carbon and energy [101,105].

Only glycerol can be converted into 1,3-propanediol by anaerobic fermentation due to its great specificity [105]. During the fermentation of *Clostridium* sp., the formation of acetic and butyric acids occurs, which interfere in the pH of the medium pH, making it more acidic. Without the proper pH control during the fermentative process, the pH of the medium reaches values below the pK_as of the acids generated, leading to their dissociated forms. With this, the dissociated acids permeate the cell membrane and remain in the associated form within the intracellular fluid (more alkaline region), without being able to cross the membrane, causing an imbalance by the energy expended for the maintenance of the intracellular pH; that is, causing inhibition of cell growth and production of 1,3-propanediol [107]. However, acetic acid has an important role as a reduction equivalent in the formation of 1,3-propanediol, and the complete conversion of glycerol to 1,3-propanediol is impossible due to this need. Meanwhile, the conversion of glycerol into butanol or ethanol by *C. pasteurianum* does not suffer from the influence of by-products, because all hydrogen carriers are regenerated in the metabolic pathway [107].

3.2. Molasses

Molasses is a syrupy material obtained during the sugar production process. Industrial sugar production starts by extracting sugarcane or beet juice, which is then clarified and concentrated. The concentrated juice is subjected to successive crystallizations, followed by separation of sucrose crystals by centrifugation. The remaining viscous liquid is molasses [108,109].

Molasses composition varies a great deal among batches. Usually this by-product of sugar production is composed of water, approximately 45–50% (*w/w*) total sugars (sucrose, glucose, and fructose), suspended colloids, heavy metals, vitamins, and nitrogenous compounds [110]. Due to its composition, animal feed and fertilizer are possible destinations for this viscous liquid. However, ethanol production by fermentation is one of its main applications [109].

As it contains a high quantity of sugars, molasses is an inexpensive renewable carbon source for several bioprocesses. Since a carbon source is necessary in relatively higher concentrations compared to other medium components, molasses is a good option to reduce raw material cost [109,111]. Molasses can be quite inhibitory to several organisms due to its salinity and osmolarity, and the presence of toxic elements, such as aluminium and sulphites, and thermal sugar degradation compounds [109].

In spite of molasses being mainly used for fuel ethanol production by fermentation, other potential molecules can be obtained by fermentation of this by-product. The most promising bioprocesses using molasses as substrate for *Clostridium* fermentations is acetone-butanol-ethanol (ABE) production. Considering the importance of butanol, studies for optimization of butanol production have been undertaken. Syed et al. [112] achieved high butanol production using blackstrap molasses as substrate. The authors compared butanol production by *C. acetobutylicum* PTCC-23 to other mutant strains obtained by UV exposure, N-methyl-N-nitro-N-nitrosoguanidine and ethyl methane sulphonate treatments. Li et al. [110] achieved a similar butanol concentration from cane molasses using *C. beijerinckii* mutant obtained by combined low-energy ion beam implantation and N-methyl-N-nitro-N-nitrosoguanidine induction.

While *C. acetobutylicum* and *C. beijerinckii* are the most encouraging species due to their ability to produce butanol from molasses [113], the literature reports high butyric acid production from molasses using *C. tyrobutyricum* species [114]. Li et al. [82] improved butanol production by coculture of *C.*

beijerinckii and *C. tyrobutyricum*, since *C. tyrobutyricum* produces butyric acid from molasses increasing the butyrate availability to butanol production by *C. beijerinckii*.

In addition, several studies evidenced that molasses is a rich source of antioxidants, such as phenolic compounds. Among the phenolic compounds present in molasses it is possible to find the flavonoids, which show high antioxidant activity [115,116]. The availability and cost of sugar molasses make it an attractive feedstock to be used in many countries as raw material for fermentations, and also as a potential extra source of antioxidants, which are considered value added chemicals.

3.3. Corn Steep Liquor

Corn steep liquor (CSL) is a multicomponent and multiphase matrix, obtained as a major by-product of the corn wet-milling industry. It is a viscous concentrate of corn solubles containing amino acids, vitamins, minerals, and proteins. It has been used as a growth medium for *Lactobacillus intermedius* in mannitol production and for *Saccharomyces cerevisiae* in solvent production [117].

The standard medium for *Clostridium* sp. growth is basically composed of yeast extract, vitamins, minerals, trace metals, reducing agents, and the carbon source. The more expensive component is the reducing agent, followed by yeast extract. Yeast extract is a water-soluble portion produced by extracting the cells' contents from autolyzed yeast, which may cost \$1000 ton⁻¹. An alternative to replace complex nutrient sources such as yeast extract is corn steep liquor, which costs around \$200 ton⁻¹ [118].

Maddipatti et al. [118] produced 32% more ethanol by *Clostridium* strain P11 using CSL instead of yeast extract. They also produced seven times more butanol using this by-product in relation to yeast extract. CSL also diversified the product range produced by *C. carboxidivorans* and *C. ragsdalei*, with maximum concentrations of ethanol, n-butanol, n-hexanol, acetic acid, butyric acid, and hexanoic acid of 2.78 g/L, 0.70 g/L, 0.52 g/L, 4.06 g/L, 0.13 g/L, and 0.42 g/L, respectively [119].

3.4. Lignocellulosic Biomass

Lignocellulosic biomass is a rigid and fibrous material present in herbaceous energy and food crops; forestry, agricultural, and agro-industrial residues; municipal solid waste; and waste streams from the pulp and paper industry [120]. This renewable and abundant feedstock represents a key opportunity towards a more sustainable economy, especially due to its capacity of sequestering carbon to the soil, which is not released to the atmosphere upon harvesting [121,122]. Biofuels, biomolecules, and biomaterials produced from lignocellulosic materials present a promising alternative to crude oil, oil products, and fossil fuels due to their renewability, biocompatibility, biodegradability, and reduction in greenhouse gas emissions to the atmosphere [123,124].

This biomass is mainly composed of three different biopolymers: cellulose (35%–50%), hemicellulose (20%–35%) and lignin (10%–25%), for which proportions depend on the feedstock origin, species, harvesting time, and growth stage [120,121,125]. Acetyl groups, minerals, phenolic substituents, and ash are other constituents that can be found in this biomass [120,121]. Cellulose is a linear microfibril formed by D-glucose subunits linked through β -1,4 glycosidic bonds and has cellobiose as a repetitive unit [121,126]. Its cohesive structure is assembled by intramolecular and intermolecular hydrogen bonds and is 85% crystalline and 15% amorphous [121,127]. Cellulose is embedded in hemicellulose and lignin. Hemicellulose is a branched and amorphous structure containing different heteropolysaccharides (xylan, glucomannan, arabinoxylan, etc.), which are composed of pentose (D-xylose and L-arabinose) and hexose (D-glucose, D-galactose, and D-mannose) sugar units [121,124–126]. In addition, hemicellulose has organic acids (acetic and glucuronic acids) in its structure, whose predominance is dependent on its origin (angiosperms vs. gymnosperms). The hemicellulose of angiosperms is highly acetylated. Upon pretreatment, these acetyl groups partially catalyze the hydrolysis of hemicellulose. Lignin is an amorphous and hydrophobic polymeric structure composed of the alcohols p-coumaryl, coniferyl, and sinapyl, and is responsible for the biomass's tough structure, impermeability, and resistance to microbial and oxidative attacks [7,121,125]. This

phenolic macromolecule displays variations in both chemical composition and structure due to a low degree of order and a high level of heterogeneity.

Different routes can be considered to transform lignocellulosic biomass into important biofuels and biochemicals, which elucidate the importance of this feedstock in biorefineries. Table 2 summarizes processes and end-products commonly obtained using lignocellulosic biomass as a feedstock in three conversion routes. The thermo-chemical, biochemical, and hybrid routes use lignocellulosic materials by chemical and biochemical processes, generating important products and building blocks for the chemical and pharmaceutical industries. However, one of the main concerns regarding lignocellulosic biomass is the food crop competition between energy and food production, which is a reality in the production of first-generation biofuels from sugar and vegetable oil [3,124]. Therefore, the production of second-generation biofuels is an alternative to produce ethanol, biodiesel, and other products from non-food crops, such as agricultural and agro-industrial residues, and forestry and energy crops [3]. This leads to valuable biochemical and biofuel production from less valuable feedstock, mainly in the form of residue. The use of residues as feedstock improves the sustainability of the process by reducing residue accumulation in the environment and, therefore, reducing many problematic effects, such as greenhouse gas emissions in landfills and dumps, and contamination of groundwater [3,128].

Table 2. Conversion routes, processes, and end products using lignocellulosic biomass as feedstock.

Conversion Route	Process	End Products
Thermo-chemical	Combustion	Heat and power
	Gasification	Hydrogen, alcohol, olefins, gasoline, diesel
	Liquefaction	Hydrogen, methane, oils
	Pyrolysis	Hydrogen, olefins, oils, specialty chemicals
Biochemical	Fermentation	Second-generation fuels (ethanol, butanol), chemicals and biochemicals
	Anaerobic digestion	Methane, fertilizer (digestate)
Hybrid [7]	Pyrolysis + fermentation	Ethanol, organic acids (butyric acid, acetic acid), 2,3 butanediol

Adapted from [124].

In order to use all components of the lignocellulosic biomass, as in the case for second-generation biofuels, a chemical and/or enzymatic pre-treatment of the feedstock is necessary in order to access the cellulose embedded in lignin and hemicellulose. Physical pretreatment, such as milling, irradiation, and extrusion, are commonly used to increase the surface area of the biomass and reduce its crystallinity to improve the enzymatic hydrolysis of its components [124]. Catalyzed and non-catalyzed steam explosion, ammonia fiber explosion (AFEX), liquid hot water, and microwave-chemical are examples of physico-chemical pretreatments [124]. Hydrothermal processing has also been assessed in literature to separate lignocellulosic biomass into cellulose, hemicellulose, and lignin in a biorefinery concept [129]. Common to the paper industry, chemical pretreatments encompass the use of acid and alkaline compounds, ionic liquids, and hot water at a controlled pH to remove lignin and hemicellulose in order to decrease the lignocellulosic material polymerization degree and crystallinity, and enhance biomass porosity [124].

Cellulases and hemicellulases can be used to convert cellulose and hemicellulose to fermentable sugars [121,124]. Bagasse, straws, and fibers have an interesting quantity of C5 and C6 sugars that can be used to produce second-generation biofuels and other chemicals in the context of a biorefinery. Although the pretreated hydrolyzed lignocellulosic material is commonly used to produce ethanol by yeasts such as *Saccharomyces*, *Kluveryomyces*, *Debaryomyces*, *Pichia*, and *Zymomonas*, these materials can also be used in ABE fermentation by *Clostridium* species [121,124]. Moreover, it is possible to produce not only ethanol but also butanol, which is a promising liquid fuel. Butanol-producing *Clostridium* sp. can uptake a wide range of hexoses, pentoses, and oligomers obtained from the hydrolysis of cellulose and hemicellulose [130]. Corn fiber hydrolysate was used for *C. beijerinckii* fermentation resulting in the production of 9.3 g/L ABE [131]. The same species was used to convert the wheat straw steam-exploded hydrolysates, yielding 128 g ABE/kg wheat straw [132]. Seven grams per liter of

butanol were produced from concentrated sugar maple hemicellulosic hydrolysate by *C. acetobutylicum* ATCC824 [133].

Even though lignin is recalcitrant to micro-organisms, it can play an important role through chemical conversions leading to the production of methanol and BTX (benzene-toluene-xylenes) phenols and phenolics [121,134].

The thermochemical conversion of lignocellulosic biomass involves the use of heat and catalysts, while in the biochemical conversion organic matter is converted by micro-organisms through a series of metabolic reactions. The thermochemical–biochemical conversion or hybrid route merges these two processes, converting biomass to a gaseous substrate using thermal energy, and the resulting gas is converted to bioproducts using bacterial cultures. The hybrid route consists of the fast pyrolysis or gasification of lignocellulosic biomass producing synthesis gas (syngas), which is later converted to biofuels such as ethanol, butanol, butyrate, and 2,3-butanediol, among others [8,14,16,135–137]. In this way, all components of the lignocellulosic material are converted to synthesis gas and later fermented by *Clostridium* bacteria, overcoming the recalcitrant characteristic of this biomass and eliminating high costs related to the enzymatic pretreatment [7]. Additionally, the adoption of this hybrid strategy for processing lignocellulosic biomass allows a more efficient use of it since the whole biomass content (including lignin) can be converted to fuels and chemicals, not only the polysaccharide fractions as in the biochemical route. Nonetheless, process integration and optimization need to be done to make these processes attractive from an industrial standpoint.

3.5. Syngas

Synthesis gas, or syngas, is a gaseous mixture mainly composed of CO, H₂, and CO₂ that can present smaller quantities of methane (CH₄), hydrogen sulfide (H₂S), and nitrogen (N₂) depending on the biomass composition and conversion conditions [7]. Different biomass sources, such as municipal solid waste, animal slurry, agricultural residue, energy crops, and coal can be used to produce syngas by pyrolysis or gasification [7]. Fast pyrolysis of biomass occurs in an oxygen-free environment with moderate temperatures, resulting in syngas, biochar, and bio-oil, an energy rich liquid composed of carboxylic acids, sugars, alcohols, aldehydes, esters, ketones, aromatics, and furans [7,138]. Bio-oil and synthesis gas composition depends on the pyrolysis technology implemented and biomass composition [7]. Reactors used in fast pyrolysis, commonly fluidized bed reactors, must have a controlled temperature, high heat transfer rates, and rapid cooling of vapors [138].

Biomass can be also converted to synthesis gas through gasification, which is conducted at higher temperatures than pyrolysis (800–1000 °C), promoted by heat and/or electricity, and in the presence of a gasifying agent [7,138,139]. Synthesis gas is the main product of this thermochemical process, and biomass composition has an important impact on gas proportion and content, which can have trace amounts of sulfur (H₂S), hydrogen chloride (HCl), alkali metals (potassium and sodium), tars, and ammonia (NH₃) [7,139]. These contaminants can interfere in the microbial conversion of synthesis gas and, therefore, purification technologies should be implemented, such as wet scrubbing or hot/cold/warm gas clean up [139]. Biomass properties (ash, moisture, particle size), gasifying agent (air, steam, pure O₂), reactor type (updraft gasifier, downdraft gasifier, fluid-bed gasifier) and operational conditions (temperature, fuel:gasifying agent ratio) also influence the synthesis gas composition [136,139].

Synthesis gas conversion can produce important chemicals and fuels through the Fischer–Tropsch (chemical) process or fermentation (biochemical process) [7]. The Fischer–Tropsch process involves catalyzation by iron, cobalt, or ruthenium, producing alcohols and liquid hydrocarbons at 200–350 °C, a fixed H₂:CO:CO₂ ratio, and a limited amount of impurities [140]. The chemical conversion process has several drawbacks due to its catalytic and operational nature, including low catalyst specificity, high energetic demand, sensitivity to toxic gases and high pressure, and temperature conditions [141–143]. The fermentation of synthesis gas obtained by the pyrolysis/gasification of biomass is considered a hybrid route since it is a combination of two conversion processes: thermochemical

and biochemical [144]. Microbial conversion of syngas occurs at a determined temperature and pH, and can be performed by different acetogenic microorganisms (*C. ljungdahlii*, *C. autoethanogenum*, *Acetobacterium woodii*, *C. carboxidivorans* P7, *C. ragsdalei* P11, *Archaeoglobus fulgidus*, etc.) and hydrogenic microorganisms (*Rhodospirillum rubrum*, *Desulfotomaculum carboxydivorans*, *Thermococcus onnurineus* NA1, etc.) producing acetate, butyrate, ethanol, 2,3-butanediol, lactate, and others [7,14,17,136,145].

Synthesis gas fermentation has many advantages when compared to well-established processes in the chemical industry. Considering the Fischer–Tropsch process, syngas fermentation presents a higher tolerance to sulfur compounds; a variety of CO, H₂, and CO₂ ratios in synthesis gas that can be used as a substrate and not a fixed ratio; lower operational pressure and temperature, which decreases operational costs; and high productivity and product uniformity [8]. Regarding other biotechnological conversions of lignocellulosic biomass, synthesis gas fermentation eliminates a complex pre-treatment stage, which would enhance operational cost due to enzyme acquisition [7]. Moreover, all components of lignocellulosic biomass are converted, biomass composition does not interfere in the gas composition obtained through pyrolysis or gasification, and the resulting H₂:CO:CO₂ ratio does not affect fermentation [146].

However, one of the major bottlenecks in this process, especially concerning commercialization, is the mass transfer between gas and liquid phases due to the low solubility of synthesis gas [147–150]. The increase of gas solubility in culture media may enhance the availability of gaseous substrate to cells, improving both the cell's autotrophic growth and product conversion [147]. For micro-organisms to convert the gaseous substrate into fuels and chemicals, it is important that the nutrient is internalized by the cell, which occurs in a certain pathway from the interior of the gas bubble to the cell cytoplasm [151]. Although mass transfer can present many resistances during this pathway, most may be neglected in most bioreactors except for the resistance near the gas–liquid interface, which is a function of gas diffusivity in the liquid phase, as well as the film thickness [152]. The gas adsorption in the liquid phase is a limiting step for gas–liquid mass transfer and is accounted for in the overall volumetric mass transfer, $k_L a$, which is increased when the gas mass transfer to the liquid phase increases [152]. Consequently, productivity would increase in gas–liquid systems.

Many approaches have been proposed in literature in order to increase mass transfer in gas–liquid systems, such as gas specificity, increasing operational pressure, increasing gas and liquid flow rates, larger specific gas–liquid interfacial areas, different reactor configurations, different liquid phases, innovative impeller designs, mathematical approaches, and more [146,153–158]. Table 3 summarizes some approaches reported in literature so far. Recently, the use of perfluorodecalin (PFC) and Tween[®] 80 improved carbon monoxide mass transfer to a liquid phase composed of distilled water, PFC, and Tween[®] 80 in a stirred tank reactor with a sparger and Rushton-type and Smith-type impellers [157].

Synthesis gas obtained from residues is a cheap feedstock that can be used to produce sustainable biofuels and chemicals [136]. An ethanol productivity of 7.3 g/L.day was achieved in 5 L bioreactors during a two-stage continuous fermentation of synthesis gas using *C. ljungdahlii* [26]. Gaseous waste streams from the steel industry, with different CO, CO₂, and H₂ ratios, could be fermented by *C. ljungdahlii* and *Clostridium autoethanogenum* to produce ethanol, integrating a biorefinery and the steel industry [159]. Commercially, LanzaTech collaborated with Concord Blue Energy to produce ethanol and 2,3-butanediol by the fermentation of high quality synthesis gas obtained via gasified municipal solid wastes and agricultural residues [7]. INEOS New Planet Bioenergy also produces ethanol from syngas obtained through the gasification of vegetative waste and agricultural biomass, and in 2008 had a production rate of 100 gallons of ethanol per dry ton of feedstock using *C. ljungdahlii* [7,15]. Coskata Inc. developed bacteria for ethanol production, *Clostridium coskatii*, and have been investing in syngas fermentation for ethanol production from wood chips and waste and reformed natural gas [15].

Table 3. Mass transfer related to synthesis gas fermentation reported in literature using water as the liquid phase.

Reactor	Agitation (rpm)	Specific Gas Flow Rate (vvm)	Microorganism	Gas	$k_L a$ (h^{-1})	Ref
BCR	n/a	0.4	n/a	CO	72.0	[160]
CHF	n/a	n/a	n/a	CO	85.7–946.6	[146]
CSTR	300	n/a	<i>C. ljungdahlii</i>	CO	14.9	[161]
CSTR	400	n/a	<i>C. ljungdahlii</i>	CO	21.5	[161]
CSTR	400	0–0.32	n/a	Syngas	38.0	[162]
CSTR	500	n/a	<i>C. ljungdahlii</i>	CO	22.8	[161]
CSTR	600	n/a	<i>C. ljungdahlii</i>	CO	23.8	[161]
CSTR	700	n/a	<i>C. ljungdahlii</i>	CO	35.5	[161]
GLR	n/a	1.67	n/a	CO	129.6	[154]
HFMBR	n/a	0.625	n/a	CO	1096.2	[23]
HFMBR	n/a	0.029	n/a	CO	385.0	[163]
HFR	n/a	0.037	n/a	CO	420	[158]
MBR	n/a	0.00625–0.0625	n/a	Syngas	450.0	[154]
PBC with microbubble sparger	n/a	0–0.021	<i>R. rubrum</i>	Syngas	2.1 for CO	[148]
STR	300	0–0.032	<i>C. ljungdahlii</i>	Syngas	35.0 for CO	[148]
STR	300	0–0.032	SBR mixed culture	Syngas	31.0 for CO	[148]
STR	300	2.7	n/a	CO	166.09 ± 13.15	[157]
STR	400	0.14–0.86	n/a	CO	10.8–155.0	[164]
STR	400	0.70–2.14	n/a	CO	72.0–153.0	[155]
STR	400	0.36–1.07	n/a	CO	72.0–122.4	[165]
STR	400	0–0.032	<i>R. rubrum</i>	Syngas	101.0 for CO	[148]
STR	450	0–0.032	<i>R. rubrum</i>	Syngas	101.0 for CO	[148]
STR	500	0.36–1.07	n/a	CO	129.6–144.0	[165]
STR	500	2.0–2.7	n/a	CO	11.48 ± 57.79–399.06 ± 26.80	[157]
STR	600	0.36–1.07	n/a	CO	147.6–208.8	[165]
STR	650	0.36–1.07	n/a	CO	172.8–252.0	[165]
STR	700	0.36–1.07	n/a	CO	187.2–288.0	[165]
STR with microbubble sparger	300	n/a	SBR mixed culture	Syngas	104.0 for CO	[148]
TBR	n/a	0–0.021	n/a	Syngas	22.0	[162]
TBR	n/a	0–0.021	<i>C. ljungdahlii</i>	Syngas	137.0 for CO	[148]
TBR	n/a	0–0.021	<i>R. rubrum</i>	Syngas	55.5 for CO	[148]
TBR	n/a	0–0.021	SBR mixed culture	Syngas	121 for CO	[148]

Where: n/a—not applicable; SBR—sulfate reducing bacteria; N—impeller speed; QCO—specific gas flow rate; $k_L a$ —overall volumetric mass transfer coefficient; BCR—bubble column reactor; STR—stirred tank bioreactor; CSTR—continuous stirred tank bioreactor; HFMBR—hollow fiber membrane bioreactor; CHF—composite hollow fiber membrane; HFR—hollow fiber reactor; MBR—membrane bioreactor; PBC—packed bubble column; TBR—trickle bed reactor/GLR—gas-lift reactor; Ref—references.

4. Biomolecules Produced by *Clostridium* sp.

4.1. Ethanol

Ethanol, often referred to as ethyl alcohol or simply alcohol, is a volatile, biodegradable, low-toxicity, flammable, and colorless liquid at room temperature [166]. Global ethanol production has grown considerably over the past decade. In 2017, its global production was approximately 98 billion liters [167]. Ethanol is a very versatile building block for industry. It can be used to generate chemicals such as ethylene, propylene, 1,3-butadiene, and hydrocarbons, as well as in the production of oxygenated molecules, such as acetaldehyde, butanol, acetic acid, acetone, and dimethyl ether [168,169]. Ethylene, the largest-volume petrochemical produced worldwide, is used to produce ethylene glycol, propylene oxide, ethylene oxide, acrylonitrile, and polyethylene (PE) [170].

Only about 7% of ethanol produced in the world is made by a petrochemical process through the hydration of ethylene, and the main producers by this route are Germany, South Africa, and Saudi Arabia [171]. The vast majority of ethanol is produced by a fermentation process, using renewable sources as feedstock and microbial catalysts. Two main types of crops are used in the ethanol production industry by a well-established technology: sugar rich crops, such as sugar cane, and amyloseous crops, such as corn. Synthetic ethanol processing is economically less attractive than fermentation

due to the high production cost of ethylene and the great availability of agricultural products and byproducts [171].

Clostridium species can produce ethanol from several renewable sources, such as sugary or starchy materials (sugar cane, sugar beet, corn, etc.), lignocellulosic materials (e.g., cane bagasse and corn stover), as well as from gaseous substrates, such as CO, CO₂, and H₂. Table 4 shows some examples.

Table 4. Recent studies reported about ethanol production by *Clostridium* species.

Feedstock	Microorganism	Reactor	P _{Et} ¹ (g/L)	Q _{Et} ² (g/L.h)	Ref. ³
Syngas	<i>C. carboxidivorans</i> P7	HFM-BR	23.93	0.14	[23]
Syngas	<i>C. rasgdalei</i> P11	STR	25.26	0.001	[172]
Syngas	<i>C. ljungdhalli</i> ERI-2	CSTR/Bubble column	19.73	0.37	[26]
Syngas	<i>C. ljungdhalli</i> PETC	CSTR	19	0.30	[173]
Syngas	<i>C. carboxidivorans</i> P7	h-RPB	7	0.28	[174]
Syngas	<i>C. ljungdhalli</i>	Batch	0.49	0.03	[175]
Syngas	<i>C. carboxidivorans</i> P7	Batch	2	0.02	[176]
CO	<i>C. autoethanogenum</i> DSM 10061	Continuous gas-fed	7.14	-	[177]
Glucose	<i>C. saccharoperbutylacetonium</i> pSH2	Batch	7.9	0.11	[178]
Glucose	<i>C. acetobutylicum</i> hbd::int(69)	Fed-batch	33	0.5	[38]
Glucose	<i>C. carboxidivorans</i> P7	Batch	2.34	0.01	[179]
Lignocellulose	<i>C. thermocellum</i> ATCC31924	Batch	2.45	0.02	[180]
Lignocellulose	<i>C. cellulolyticum</i> H10	Batch	2.5	-	[41]
Lignocellulose and starch	<i>C. acetobutylicum</i> NBRC13948	SHF	1.7	0.018	[180]
Lignocellulose and starch	<i>C. acetobutylicum</i> NBRC13948	SSF	1.5	0.01	[180]
Cellobiose	<i>C. thermocellum</i> LL1275	Batch	5	0.07	[63]
Cellobiose	<i>C. cellulovorans</i> 83151-adhE2	CBP	2.03	0.03	[181]
Cellobiose	<i>C. phytofermentans</i> ATCC700394	CBP	7	0.03	[36]
Glycerol	<i>C. pasteurianum</i> MTCC6013	Immobilized cells	1.94	0.01	[182]

¹ P_{Et}: ethanol production; ² Q_{Et}: ethanol productivity; ³ Ref.: reference.

Fernandez-Naveira et al. [180] reported an ethanol production of 2.34 g/L from a glucose (30 g/L) fermentation process by *C. carboxidivorans* DSM 15,243 in a continuous bioreactor. A mutant strain of *C. acetobutylicum* ATCC824, developed by the disruption of the butyrate/butanol pathway, produced 33 g/L of ethanol in a fed-batch fermentation process using glucose as a substrate with a productivity of 0.5 g/L.h [38]. In another study, *C. saccharoperbutylacetonicum* N1-4 had some genes overexpressed to develop a more robust strain, which resulted in a 400% increase in ethanol production. Glucose (80 g/L) was used in a batch bioreactor process and 7.9 g/L of ethanol was produced after 72 h of fermentation [178].

Khanna et al. [178] reported the bioconversion of crude and pure glycerol into ethanol, butanol, and 1,3-propanediol by immobilized *C. pasteurianum* MTCC 6013, using a silica gel chromatography column (80–120 mesh grade) as immobilization support. Pure glycerol (25 g/L) formed more products (19 g/L) than crude glycerol (12 g/L). In these conditions, 1.94 g/L of ethanol, 9.23 g/L of 1,3-propanediol, and 7.73 g/L of butanol were obtained [182].

Ethanol can also be produced from lignocellulosic materials, which is commonly called second-generation ethanol. Agricultural residues, such as sugar cane bagasse and corn stover, grasses, such as switchgrass, and forestry and wood residues have been studied in order to develop a cost-effective second-generation process using these plentiful feedstocks [169,183]. In a very recent study, Singh et al. [180] reported an ethanol production of 2.45 g/L during 120 h of cellulose batch fermentation (30 g/L) by *C. thermocellum* ATCC31924. *C. thermocellum* LL1275 produced 5 g/L of ethanol (productivity of 0.07 g/L.h) through batch fermentation with cellobiose as a substrate [63]. It was reported that 8.5 times more ethanol was produced by a genetic engineered strain of *C. cellulolyticum* in comparison to the wild strain, using switchgrass as feedstock in a batch fermentation process [41].

The production of ethanol with lignocellulosic biomass can be processed through four different configurations, depending on the feedstock, the microorganism chosen, and the product desired. These configurations are known as SHF (separate hydrolysis and fermentation), SSF (simultaneous saccharification and fermentation), SSCF (simultaneous saccharification and co-fermentation), and CBP

(consolidated bioprocessing) [184]. In the SHF, the hydrolysis of the biomass and the hexose/pentose fermentations are carried out in separate reactors. The main advantage of this configuration is the possibility of performing each step in its optimal conditions of pH and temperature. However, high concentrations of glucose and/or cellobiose in the first step inhibit the cellulases and reduce their efficiencies. The SSF configuration occurs with cellulose hydrolysis and hexose fermentation in the same bioreactor, reducing the inhibition of cellulases by end products as observed in the SHF, but making it difficult to process both steps in their optimum conditions of pH and temperature. Furthermore, microorganisms used in the SSF are able to ferment only glucose, not being able to utilize xylose. The SSCF configuration is similar to the SSF, but glucose and xylose can be fermented in the same bioreactor, as some genetically engineered strains are developed to ferment both substrates [185,186]. CBP configuration is represented by one single microorganism that produces enzymes to hydrolyze cellulose and hemicellulose, and converts the resulting sugars into ethanol. Thus, the enzyme production, hydrolysis, and fermentation steps occur in the same bioreactor, reducing costs and increasing process efficiency. However, this type of biocatalyst is still in the early development stage [185,187]. In 2014, a study reported the production of ethanol and butanol through SHF and SSF processes with *C. acetobutylicum* NBRC13948. In this work, corn and wood chips of *Quercus acutissima* were used as feedstock and 1.7 g/L of ethanol was produced after 96 h of fermentation in the SHF process, while in the SSF process 1.5 g/L of ethanol was obtained after 144 h [188]. *C. phytofermentans* was used in a CBP configuration with corn stover as feedstock. An ammonia fiber expansion (AFEX) pretreatment process was conducted and 7 g/L of ethanol was produced after 264 h of fermentation [36].

Another way to transform the lignocellulosic biomass into ethanol is the hybrid technology that involves thermochemical and biochemical steps, known as syngas fermentation [182], as already mentioned in the feedstock section. In addition to the syngas produced from gasification of biomass, industrial waste gas streams containing CO, H₂, and CO₂ can also be converted to ethanol by *Clostridium* species, in the biochemical step [184]. The main advantage of the syngas fermentation process is that all components of lignocellulosic material, including lignin, are converted into syngas and later fermented, overcoming the recalcitrant characteristic of this biomass and eliminating the elevated costs related to the enzymatic pretreatment step [7]. In 2014, it was reported that 24 g/L of ethanol was obtained from syngas fermentation (50% CO, 30% H₂, and 20% CO₂) by *C. carboxidivorans* P7 in a hollow fiber membrane biofilm reactor (HFM-BR) [23]. Richter et al. [26] reported a two-stage syngas fermentation process by *C. ljungdallii* ERI-2, operating one bioreactor for cell growth and a bubble column bioreactor equipped with a cell recycle module for ethanol production. Syngas containing 60% CO, 35% H₂, and 5% CO₂ was used, and 18 g/L of ethanol was obtained, with a productivity of 0.37 g/L.h. A study carried out recently with *C. carboxidivorans* P7 reported an ethanol productivity of 0.28 g/L.h with a syngas composition of 20% CO, 5% H₂, 15% CO₂, and 60% N₂, in a horizontal rotating packed bed biofilm reactor (h-RPB). Seven grams per liter of ethanol were obtained using the h-RPB reactor, which was 3.3 times higher than that obtained in a CSTR (continuous stirred tank reactor) under the same conditions [189].

4.2. Butanol

Butanol (butyl alcohol) is a four-carbon alcohol with the molecular formula C₄H₉OH. It is a volatile, biodegradable, low-toxicity, flammable, and colorless liquid at ambient temperature. There are four isomeric structures of butanol: n-butanol, sec-butanol, isobutanol, and tert-butanol. This alcohol is an important building block for the chemical industry, being used as an intermediate to the production of methacrylate esters, butyl acrylate, butyl glycol ether, butyl acetate, and plasticizers. It can also be used as a diluent for brake fluid formulation, and for the production of antibiotics, vitamins, and hormones [190,191]. One of the most important applications of butanol is as a direct replacement of gasoline or as a fuel additive, due to the low vapor pressure and corrosivity, which allows its transportation and storage in the same infrastructure existing for gasoline, as well as its blend with existing gasoline at much higher proportions than ethanol [191,192]. Furthermore, as butanol is a

four-carbon alcohol, it has the double carbon content of ethanol and contains 25% more energy [191]. These advantages make butanol outstrip ethanol as an alternative biofuel [193].

Butanol has been traditionally produced by anaerobic fermentation of sugar-rich substrates using *Clostridium sp.* via ABE fermentation process. With the emergence of the petrochemical industry in the 1950s, most butanol in the world started to be produced from fossil oil, through the hydroformylation of propene, a process known as oxo synthesis [190]. In the 1970s, the global energy crisis rekindled the worldwide focus towards development of alternative fuels, reigniting interest in ABE fermentation [190,194]. The fermentation technology of butanol production by *Clostridium sp.* has some drawbacks, such as the relatively high substrate cost arising from the use of edible biomass, the low final butanol concentration obtained (less than 20 g/L), the low butanol selectivity, the low volumetric butanol productivity (less than 0.5 g/L.h), and the high cost of recovery. Thus, butanol fermentation is less competitive than that of other biofuels [193]. Aiming at overcoming these disadvantages related to the fermentation technology, some studies reported the development of genetically engineered strains to improve butanol yield, different modes of operation to increase the productivity, different recovery processes, and the use of non-edible feedstocks focusing on residual biomass [193].

Lignocellulosic biomass, syngas, molasses, and glycerol are promising feedstocks for ABE fermentation. Qureshi et al. [29] achieved 34.77 g/L of butanol using corn stover hydrolysate, and 30.86 g/L using barley straw hydrolysate, as substrates in the fermentation process by *C. beijerinckii* P260, with simultaneous product recovery. Using crude glycerol (50 g/L) as a substrate, butanol production of 8.95 g/L was reported in a batch fermentation with *C. pasteurianum* DSM 525, and 0.119 g/L.h of productivity was achieved [195]. A fibrous bed reactor with gas stripping recovery was used to produce butanol with cassava bagasse hydrolysate by a hyper-butanol-producing *C. acetobutylicum* strain (JB200). More than 76 g/L of butanol was obtained with a productivity of 0.32 g/L.h [196]. Only 1 g/L of butanol and 3 g/L of ethanol were produced after 600 h in batch fermentation (70% CO, 20% H₂, 10% CO₂) using *C. carboxidivorans* P7 [197].

Mutagenesis and metabolic engineering techniques have been used to develop new strains capable of producing butanol in adverse situations that the wild strain could not. Zhang et al. [198] achieved a butanol productivity of 0.53 g/L.h using a metabolically engineered strain of *C. tyrobutyricum* to ferment sugar cane juice, with corn steep liquor as the nitrogen source, using a fibrous bed bioreactor (FBB) in a repeated batch mode for 10 consecutive cycles in 10 days. A butanol concentration of 12.8 g/L was detected [196]. Lee et al. [37] reported the development of a metabolically-engineered strain of *C. acetobutylicum* that produced 44.6 g/L of butanol. Glucose was used as the substrate in a continuous fermentative process, and in situ adsorptive recovery of butanol. A butanol productivity of 2.64 g/L.h was reported, which represents a high value compared to similar studies.

Different fermentation techniques can be adopted in the ABE process, including: batch, fed-batch, semi-continuous, free cell continuous, immobilized cell continuous, cell recycle cell, biofilm reactor, fed-batch extractive fermentation, and fed-back with in situ product recovery. The batch process is the most reported due to the low contamination risk and simple operation, but a maximum ABE solvent concentration of 25–30 g/L has been obtained [197]. Zhang et al. [198] reported an immobilized cell fermentation system with co-culturing of *C. beijerinckii* and *C. tyrobutyricum*, using hydrolysate cassava bagasse as feedstock. More than 13 g/L of butanol was produced with a productivity of 0.44 g/L.h.

Obstacles, such as the low productivity, low yield, and low substrate consumption, are a result of solvent accumulation that causes severe inhibition. Efficient recovery processes are able to overcome these drawbacks, but usually lead to high processing cost and to large quantities of disposal water. The most-used recovery technique in the industry is conventional distillation, but other techniques have been investigated in order to reduce costs, enhance productivity, eliminate inhibition effects, and increase sugar consumption. Among these, liquid–liquid extraction, pervaporation, gas stripping, perstraction, reverse osmosis, ionic liquid extraction, adsorption, and aqueous two-phase separation can be highlighted [199]. A very recent study reported a two-stage fed-batch fermentation of glucose by

C. acetobutylicum MTC11274 with magnesium limitation and calcium supplementation, integrated to a gas stripping system. More than 54 g/L of butanol was detected, with a productivity of 0.58 g/L.h [200]. Dong et al. [201] reported a ceramic hollow fiber-supported polydimethylsiloxane (PDMS) composite membrane used for the pervaporation of butanol. The results showed the PDMS composite membrane exhibited high and stable performance for butanol recovery from ABE systems. Raganati et al. [202] showed an efficient recovery of butanol from ABE fermentation broth by adsorption on Amberlite XAD-7, as this adsorbent presents a high affinity for butanol and a poor affinity for glucose.

4.3. Acetone

Acetone, also known as propanone, is a colorless, volatile, flammable, and toxic liquid. Represented by the chemical formula $(\text{CH}_3)_2\text{CO}$, it is the simplest, smallest, and commercially most-important aliphatic ketone. Almost 7 million tons of acetone are produced globally per year [203]. The development of acetone industrial production by fermentation was promoted by the outbreak of the First World War. The Weismann process was patented in March 1915 [87,204]. The main objective to produce acetone by this time was the conversion into cordite, a smokeless ammunition powder [87,204]. However, in the 1950s, routes to produce solvents from oil were made cost-competitive with fermentation. Furthermore, the major feedstock for fermentation processes was molasses, which spiked in price because of animal feed demand [204,205]. Today, approximately 100 years after the publication of the Weismann process, its modern approaches are gaining interest once more, with a different driver, namely, the search for more sustainable processes [204].

At present, the global acetone industry is driven by the solvents sector, representing approximately 34% of global demand in 2017. As a solvent, it is used in products, such as nail polish removers, cement, lacquers, cleaners, paint, coatings, films, and adhesives [206]. It is also used for MMA (methyl methacrylate) production, to form PMMA (polymethyl methacrylate), and as a fuel additive [206,207].

Acetone can be produced through different methods, such as the cumene process, from alkane nitriles, hydrolysis of geminal dihalides, dehydrogenation of isopropyl alcohol, ozonolysis of alkanes, and the fermentation process. About 96% of the world's acetone production is as a by-product of phenol by the cumene process, which uses benzene and propylene, two petrochemical products, as raw materials [206]. Integrated metabolic and evolutionary engineering was applied to *C. cellulovorans* and a 138-fold increase in butanol production was achieved [208]. Due to the current concern about greenhouse gas emissions and the replacement of petrochemical processing by renewable processes and feedstocks, fermentation technologies are of interest to academic and industrial communities [207].

The fermentation of sugar using *Clostridium* strains is a well-known industrial process, for which main products are acetone, butanol, and ethanol (ABE), obtained in a molar ratio of 3:6:1 [207]. Strictly anaerobic Gram-positive microorganisms possess a fermentation metabolism and utilize a variety of sugars, oligosaccharides, and polysaccharides by two distinct phases, acidogenic and solventogenic [209]. Usually, desired products of ABE fermentation are butanol and ethanol; acetone is the major by-product and is produced in the solventogenic phase, along with ethanol and butanol [207]. Glucose is the preferred substrate for ABE fermentation; however, a range of substrates can be used, such as simple sugars (galactose and xylose), disaccharides (maltose, sucrose, and lactose) and complex polysaccharides (cellulose and hemicellulose). Furthermore, direct conversion of starch without any hydrolysis step is possible with *Clostridium* strains [207].

The most widely used species for the production of acetone is *C. acetobutylicum*, although *C. beijerinckii* has also been used with good results. Li et al. [210] reported a fermentation process in a 7 L bioreactor with *C. acetobutylicum* ATCC824, using a cassava-based medium. Six grams per liter of acetone were produced after 50 h of fermentation. The addition of acetate in the culture medium is a new approach to increase acetone and butanol production in ABE fermentation [211,212]. Acetone production doubled with the addition of 4 g/L of acetate in the culture medium containing 50 g/L of glucose with *C. saccharoperbutylacetonicum* N1-4 [211]. For pretreated corncob fermentation by *C. beijerinckii* TISTR1461, an inferior increase (8%) was detected with acetate addition [212].

Batch is the most common operational process for ABE studies, despite its drawbacks, such as low cell density, low reactor productivity, nutritional limitations, and severe product inhibition. An important approach that has attracted interest to achieve greater conversion rates in ABE fermentation is cell immobilization technology. The immobilization can be done by adsorption or entrapment, but due to diffusion limitation problems, passive adhesion to surfaces is preferred [213]. Six grams per liter of acetone was obtained as by-product with *C. acetobutylicum* DSM792 using glucose as a substrate in a continuous column bioreactor with wood pulp as the immobilization material [213]. Kong et al. [39] reported a batch fermentation using free and immobilized *C. acetobutylicum* XY16 with modified sugarcane bagasse as a support for immobilization. A 1.4 times increase in acetone production was achieved with the cell immobilized batch process.

4.4. 1,3-Propanediol

1,3-Propanediol (1,3-PDO), also called trimethylene glycol, 1,3-dihydroxypropane, or propylene glycol, is a three-carbon molecule with the molecular formula $C_3H_8O_2$ [214]. Due to its enormous applications in the synthesis of polyesters, polyethers, polyurethanes, and heterocyclic compounds, such as indole and quinolines, 1,3-PDO is a promising bulk chemical. The chemical industry also uses 1,3-PDO to obtain resins, engine coolants, mortars, and inks [215]. The most important application involves the synthesis of a superior polymer known as polytrimethylene terephthalate (PTT). PTT is a biodegradable polyester with great potential application in textile, carpet, and upholstery manufacturing. Compared to other similar polymers, such as polyethylene terephthalate (PET), polybutylene terephthalate (PBT), or nylon, PTT has better elasticity and better modulus. It shows higher ultraviolet (UV) resistance and tends less to electrostatic charging [214,216].

A number of different chemical and fermentative routes can be used to synthesize 1,3-PDO. The chemical routes to produce 1,3-PDO are hydration of acrolein, followed by hydroformylation and hydrogenation of ethylene oxide. These processes require expensive catalysts, high temperature, and pressure, with several disadvantages, such as toxic intermediates and non-renewable raw materials [214,216]. Alternative fermentative routes are more environmentally friendly compared to chemical processes. Glycerol is the unique substrate that can be directly metabolized to 1,3-PDO. Native microorganisms that convert glycerol into 1,3-PDO belong mainly to the genera *Clostridium*, *Klebsiella*, *Citrobacter*, *Enterobacter* and *Lactobacillus*. All are able to produce 1,3-PDO using glycerol as the sole carbon source except *Lactobacillus*, which produces 1,3-PDO by co-fermentation using both sugar and glycerol as substrates simultaneously [214]. Among these microorganisms, *K. pneumoniae* and *C. butyricum* have been most intensively studied due to their substrate tolerance, high yield, and productivity. Although *C. butyricum* is strictly anaerobic and *K. pneumoniae* is facultative anaerobic, *C. butyricum* is more interesting for industrial application because *K. pneumoniae* is classified as an opportunistic pathogen [217]. Another possibility of producing 1,3-PDO involves genetically engineered strains that can utilize sugar to glycerol and glycerol to 1,3-PDO consecutively [214,216,217].

Many strategies have been investigated to increase yield and productivity of 1,3-PDO using *Clostridium* strains, including by-product reduction; substrate and product inhibition reduction; different bioreactor operation modes; mutagenesis and genetic modification of strains; cell immobilization; and co-culture fermentation [218–220]. Production of 1,3-PDO from glycerol using different *Clostridium* species and strategies is compared in Table 5.

Table 5. Production of 1,3-PDO from glycerol using different *Clostridium butyricum* strains and strategies.

Strain	FS	Strategy	Scale	C _{1,3-PDO} ¹	Y _{1,3-PDO} ²	Q _{1,3-PDO} ³	Ref. ⁴
<i>C. butyricum</i> CNCM1211	PG ⁵	B ⁷	1-L	37.1	0.53	1.32	[221]
<i>C. butyricum</i> CNCM1211	CG ⁶	B	1-L	63.4	0.57	0.63	[221]
<i>C. butyricum</i> F2b	CG	B	2-L	47.1	0.52	1.34	[222]
<i>C. butyricum</i> F2b	CG	C ⁸	2-L	44.0	0.51	1.76	[222]
<i>C. butyricum</i> VPI 3266	PG	C	2-L	29.7	0.62	2.98	[220]
<i>C. butyricum</i> VPI 3266	CG	C	2-L	31.5	0.50	3.15	[223]
<i>C. butyricum</i> AKR102a	PG	FB ⁹	1-L	93.7	0.52	3.30	[40]
<i>C. butyricum</i> AKR102a	CG	FB	1-L	76.2	0.51	2.30	[40]
<i>C. butyricum</i> AKR102a	CG	FB	200-L	61.5	0.53	2.10	[40]
<i>C. butyricum</i> DSP1	CG	B	6.6-L	37.6	0.53	1.12	[224]
<i>C. butyricum</i> DSP1	CG	FB	6.6-L	71.0	0.54	0.68	[224]
<i>C. butyricum</i> DSP1	CG	B	42-L	36.4	0.52	1.13	[224]
<i>C. butyricum</i> DSP1	CG	B	150-L	37.2	0.53	1.33	[224]
<i>C. butyricum</i> VPI1718	CG	RB ¹⁰	1-L	65.5	0.52	1.15	[225]
<i>C. butyricum</i> NCIMB 8082	CG	B	1-L	32.2	0.52	2.38	[226]
<i>C. butyricum</i> NCIMB 8082	CG	FB	1-L	29.8	0.48	2.55	[226]

¹ C_{1,3-PDO}, final 1,3-PDO concentration (g/L); ² Y_{1,3-PDO}, 1,3-PDO produced per glycerol consumed (g 1,3-PDO/g glycerol); ³ Q_{1,3-PDO}, 1,3-PDO overall productivity (g/L.h); ⁴ Ref.: reference; ⁵ PG, pure glycerol; ⁶ CG, crude glycerol; ⁷ B, batch fermentation; ⁸ C, continuous fermentation; ⁹ FB, fed-batch fermentation; ¹⁰ RB, repeated batch cultivation.

4.5. Other Biomolecules

Clostridium spp. may also produce other interesting molecules besides those already mentioned. Butyric acid, isopropyl alcohol, *n*-caproic acid, and hexanol are some of the chemicals that can also be produced by the genus *Clostridium* [198,227–230].

Butyric acid is a four-carbon volatile acid with a global market of more than 80,000 tons per year [227,231]. This carboxylic acid is an important chemical, as its derivatives present several commercial applications. For example, butyrate esters can be applied as fragrance- and flavor-enhancing agents in beverages, food, and cosmetics [227]. Due to its many health benefits, including antineoplastic effects on the large intestine and colon, butyrate and its derivatives have been widely used in pharmaceuticals as drugs for treating hemoglobinopathies, colon cancer, and gastrointestinal diseases; and as prebiotic supplements to animal feeds replacing antibiotics [232]. Another relevant application of butyric acid is butanol production by either biotransformation using microorganisms or catalytic chemical process [230,232].

At present, butanol is mainly produced by the petrochemical industry via oxidation of butyraldehyde obtained from oxosynthesis of propylene [227,232]. However, concerns regarding environmental impacts and the rising desire to use renewable resources have driven industrial attention toward fermentative production of butyric acid, especially for applications in the food and pharmaceutical industries [227,232].

Although butyric acid can be synthesized by various strains belonging to the genera of *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium*, *Megasphaera*, *Roseburia*, and *Coprococcus* [227,231,232], the preferred strains for potential commercial uses are from the genus *Clostridium* because of their higher and stable productivity and production titers [230,232]. Most butyric acid-producing clostridia ferment glucose, xylose, lactose, starch, and glycerol for cell growth and butyric acid production. Cellulose and CO₂ can also be converted into butyrate by some species [227,232]. *C. carboxidivorans* can utilize CO, CO₂, and H₂ to produce butyric acid. *C. cellulovorans*, *C. polysaccharolyticum*, and *C. populeti* can use cellulose for butyrate synthesis [232]. Some *Clostridium* species can be manipulated to produce either solvents or acids as the main products, depending on culture conditions. Several species, including *C. butyricum*, *C. tyrobutyricum*, and *C. thermobutyricum* produce butyrate as the main product with a relatively high productivity and yield; thus, they are the most studied species due to their high commercial potential for butyric acid production. *C. kluyveri*

can produce butyric acid as a major product from ethanol and acetate as substrates; however, *n*-caproic acid is produced instead of butyrate when ethanol is present in excess of acetate [227,232].

n-Caproic acid, a six-carbon chain carboxylic acid, is a versatile platform chemical for producing flavor additives for the food industry and biofuels for aviation. This molecule is also a potential antimicrobial agent that can be used as a green antibiotic [228,233]. *n*-Caproic acid is produced by chain elongation of a carboxylic acid, which is a reversed β -oxidation pathway using ethanol or lactic acid as an electron donor. Short-chain carboxylic acids are elongated by adding two carbons (acetyl-CoA derived from ethanol) each time, converting them into chemicals with six or more carbons [228,234].

C. kluyveri and *Clostridium* sp. BS-1 were identified as *n*-caproic acid producers [233]. The use of *C. kluyveri* pure culture was reported in the caprogenic processes, but open cultures are known to increase metabolite production. Studies have used different substrates, such as acetic acid with ethanol, syngas fermentation effluent with ethanol, yeast-fermentation beer, and lactic acid [233,235].

Another valuable organic molecule that can be obtained by *Clostridium* anaerobic fermentation is isopropanol, also called isopropyl alcohol. Isopropanol is an important organic solvent used in paints and varnishes, removers, antiseptic solutions, printing, perfumery, and cosmetics [236]. This molecule is also an additive for gasoline and diesel, and an important precursor for the production of green propylene [229].

Although ABE fermentations are usually conducted using *C. acetobutylicum*, several *C. beijerinckii* sp., having an additional primary/secondary alcohol dehydrogenase, can convert acetone to isopropanol. Studies using *C. beijerinckii* have reported mainly butanol and isopropanol production with very little ethanol/acetone, unlike other ABE organisms, where the ratio is 6:3:1 (butanol:acetone:ethanol weight ratio) [227,229,237].

Hexanol is also an organic solvent that can be produced by *Clostridium* autotrophic growth on CO and H₂. *C. carboxidivorans* derives energy and carbon for growth from CO and H₂, and produces ethanol, butanol, and hexanol by reduction of organic acids formed in the mechanism of energy conservation and synthesis of cell material [86,197].

5. Concluding Remarks

First-generation biorefinery processes are based on the use of sugar-containing food crops as feedstock and lead to food–fuel competition. The use of lignocellulosic biomass, waste, or waste gases increases the sustainability of biorefineries, thereby overcoming the food–fuel dilemma. These two concepts can be integrated in a biorefinery, reducing the use of food crops, with the help of *Clostridium* species, as proposed in the hypothetical scenario presented in Figure 1. In the example, because sugarcane bagasse can be used to generate fuels and chemicals, reduced amounts of sugarcane juice are needed for this purpose. In this concept, the lignocellulosic biomass (sugarcane bagasse) can be submitted to pre-treatment for enzymatic hydrolysis for microbial transformation (biochemical route), and also be thermally converted to syngas for microbial fermentation (hybrid route). In both routes, significant progress has been made. Economic and market data can be used to choose between these processes, minimizing the disadvantages related to each one. *Clostridium* species are essential in this concept, since they can use syngas for solvent and fuel production, and can also use cellulosic material directly without the need of enzymatic hydrolysis. Further research and development are essential to improve yield and productivity, and to reduce the production costs of syngas fermentation. To achieve this, strategies of developing recombinant clostridia to increase product tolerance and the use of metabolic engineering to direct carbon flow to the production of target molecules must be adopted. In addition, the design of new bioreactor configurations to circumvent inherent problems of gas–liquid mass transfer, along with process optimization and downstream integration, will result in an efficient process with greater scalability potential.

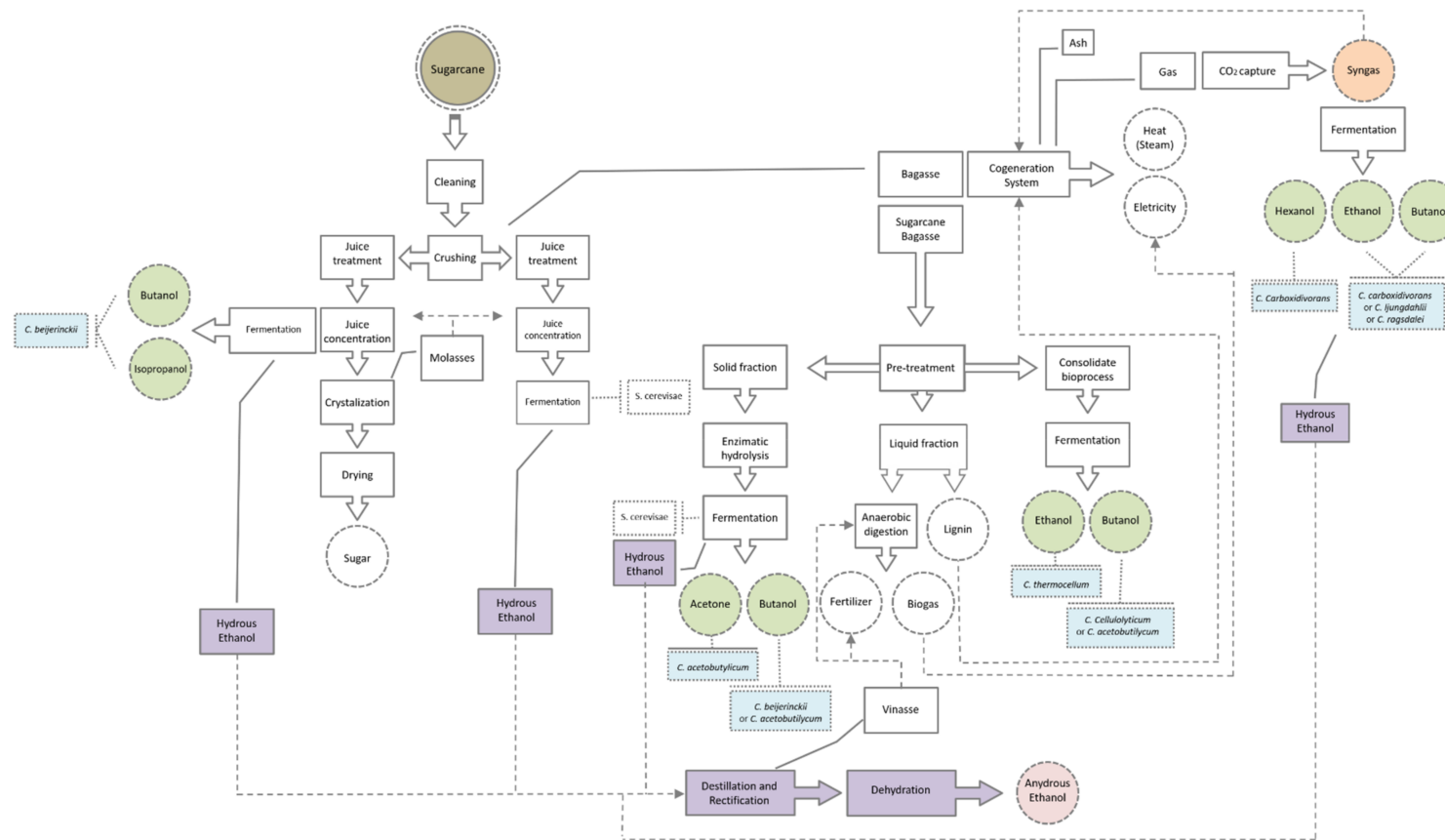


Figure 1. Schematic diagram of a sugarcane biorefinery producing ethanol, sugar, power, butanol, syngas, fertilizer, hexanol, and acetone, based on [238–241] and on information of the present review. Dotted circle—intermediate products and end products; dotted arrows—product destination from one process for use as raw material in another process; blue—*Clostridium* species; green—direct *Clostridium* metabolism products; purple—intermediate product after bioprocess using *Clostridium* species and its process for product refining; red—by-product after bioprocess using *Clostridium* species; and orange—residue used as raw material for fermentation using *Clostridium* species.

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