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Review

Thermoanaerobacter Species: The Promising Candidates for Lignocellulosic Biofuel Production

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ABSTRACT: *Thermoanaerobacter* species, which have broad substrate range and high operating temperature, can directly utilize lignocellulosic materials for biofuels production. Compared with the mesophilic process, thermophilic process shows greater prospects in consolidated bioprocessing (CBP) due to its relatively higher efficiency of lignocellulose degradation and lower risk of microbial contamination. Additionally, thermophilic conditions can reduce cooling costs, and further facilitate downstream product recovery. This review comprehensively summarizes the advances of *Thermoanaerobacter* species in lignocellulosic biorefinery, including their performance on substrates utilization, and genetic modification or other strategies for enhanced biofuels production. Furthermore, bottlenecks of sugar co-fermentation, metabolic engineering, and bioprocessing are also discussed.

Keywords: *Thermoanaerobacter*; Lignocellulose; Biofuel; Co-fermentation; Metabolic engineering; Bioprocessing

1. Introduction

Fossil fuel, consumed worldwidely, is the non-renewable energy, the exhaustion of which has inspired the interest in the environmentally friendly energy—biofuels [1,2]. The usage of biofuels such as bioethanol can reduce fossil fuel consumption, cut down the emission of SO_2 and NO_X, and consequently do good to the environment [3]. Lignocellulose is seen as an abundant, sustainable and low-cost resource on the earth for biofuels production, which can not only avoid the conflict between food and fuels, but also help to deal with the globally abundant agricultural residues to protect the local environment [4,5]. Lignocelluloses such as wheat straw, rice straw and sugarcane bagasse were adopted for biofuels production [6–8], suggesting bio-processing of biomass is widely accepted due to the good perspective [9]. Unfortunately, biofuel engineers must face the fact that cellulosic ethanol still cannot match the low cost of petro-chemicals and first-generation (1G) ethanol (usually produced from cereal grains, sugar and amylaceous plants, popularly in USA and Brazil) [5,10]. The second and third-generation ethanol is obtained from lignocellulosic materials and algae, respectively, while the latter is not well developed due to some limited factors like the technology for extracting bioethanol from seaweed [11]. The conversion of lignocellulosic feedstock into biofuels at competitive prices depends on the efficient conversion of lignocellulosic biomass. In order to achieve this purpose, changes need to be done urgently, such as reducing the enzyme costs, application of enzyme-free saccharification processes, enforcing the co-fermentation of mixed sugars for biofuels production [5,12–14].

Generally, lignocellulose mainly contained lignin (15–20%), cellulose (40–50%) and hemicellulose (25–35%) [15], which could be decomposed to monosaccharides and disaccharides then followed by biofuels conversion [16]. Traditional method for lignocellulose pretreatment aims at obtaining glucose by cellulases, including endoglucanase, exoglucanase and β-glucosidase. However, complete hydrolysis of lignocellulose faces many challenges in biomass application [17,18]: (1) β-glucosidase is the rate limiting enzyme for cellulose hydrolysis and large amount usage of β-glucosidase will increase the cost; (2) Glucose in the

hydrolysate is an inhibitor of cellulase, which leads to low efficiency of complete hydrolysis of cellulose; (3) The presence of glucose in the complete hydrolysate can not only easily cause the microbial pollution, but also induce the carbon catabolite repression (CCR), prolonging the fermentation period. Therefore, pretreatment with incomplete hydrolysate of lignocellulose began to emerge [19,20].

Among microorganisms that can ferment biomass to biofuels, thermophilic anaerobic bacteria, with an optimal growth temperature range of 45–70 °C, have some advantages over mesophilic bacteria [21], such as the ability to co-utilize glucose and xylose by *Thermoanaerobacter* species [22,23] (they are further subdivided into the genus *Thermoanaerobacter* and the genus *Thermoanaerobacterium* due to some differences such as the absence or presence of the nitrogenase genes [24]), lower risk of the contamination for high temperature fermentation [25], less heat exchange following pretreatment and higher economy to recover ethanol at temperatures over 50 °C by continuous distillation [26]. Another common and well-studied thermophile is *Clostridium thermocellum*, which could degrade cellulose but the titer of biofuels produced was low [27–29]. Here, we mainly focus on *Thermoanaerobacter* species, which might be the potential chassis microorganisms for lignocellulosic biorefinery with the ability to directly utilize hemicellulose or even cellulose [30,31]. This review will comprehensively discuss the utilization of substrates, genetic operation platforms constructed and the strategies employed for enhancing the biofuel production of *Thermoanaerobacter* species (Figure 1). Additionally, the potential of *Thermoanaerobacter* species co-cultured with *C. thermocellum* for consolidated bioprocessing (CBP) will be also addressed.

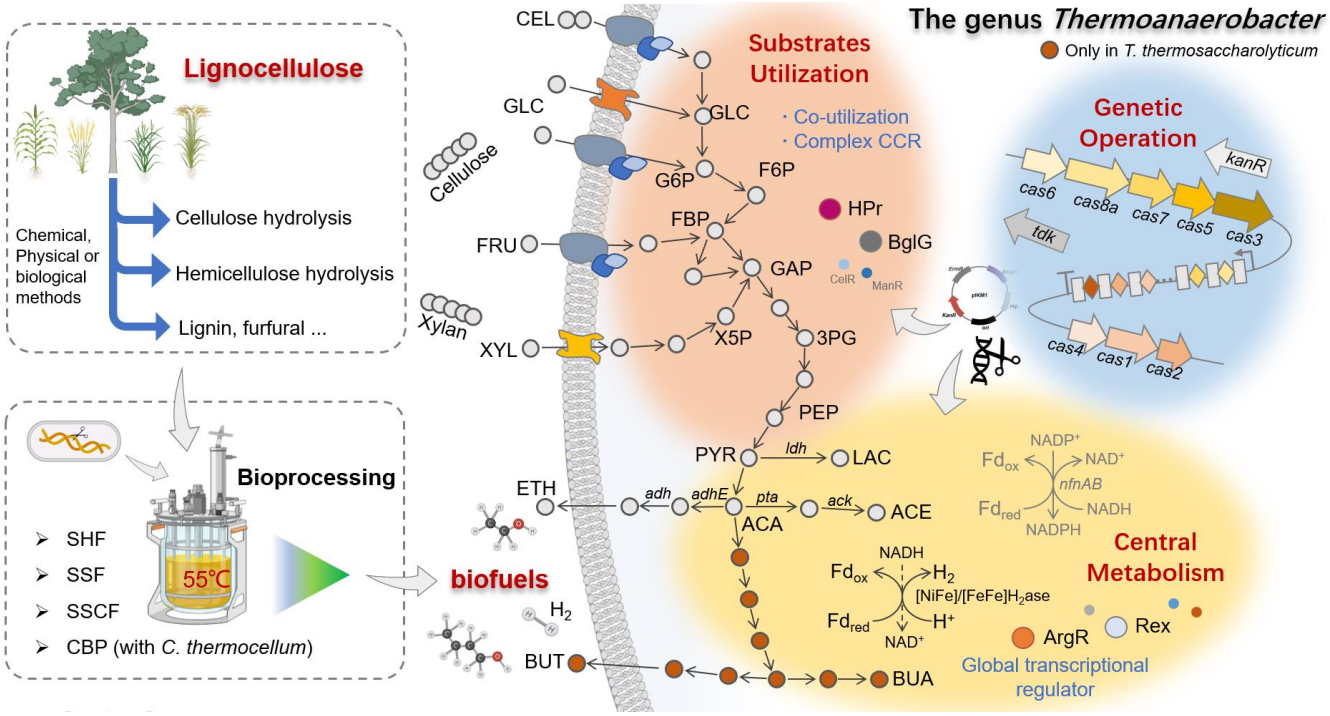

Figure 1. Diagram of different substrates utilization, genetic operation and main metabolic pathways in *Thermoanaerobacter* species for biofuels fermentation under various bioprocesses. CEL, cellobiose; GLC, glucose; FRU, fructose; XYL, xylose; ETH, ethanol; BUT, butanol; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-biphosphate; GAP, glyceraldehyde-3-phosphate; X5P, xylulose-5-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; ACA, acetyl-CoA; ACE, acetate; BUA, butyrate.

2. Excellent Performance on Substrates Utilization

2.1. Xylan and Xylose Metabolism in Thermoanaerobacter Species

Xylan is the second most abundant plant polysaccharide on the earth, and the D-xylose is the primary ingredient of the hemicellulose from lignocellulosic biomass, both of which are not the common or preferred substrates for most microorganisms [32]. Xylose metabolism appears to occur exclusively via the traditional xylose isomerase-xylulokinase-pentose phosphate pathway in *Thermoanaerobacter* species [33].

As shown in Figure 2, the genus *Thermoanaerobacter* has been reported to be an excellent utilizer of xylan and xylose [30,34], the feature of which is of particular industrial interest. In *Thermoanaerobacterium aotearoense* SCUT27 (SCUT27), results of the genome annotation revealed 10 genes related to xylose utilization, including one xylose isomerase, one xylulose kinase, and 8 ABC-type xylose transporters [35]. Compared to *T. thermosaccharolyticum* DSM571, strain SCUT27 possesses two more D-xylose ABC transporter-related genes for xylose utilization. Strain SCUT27 also can degrade xylan directly and four genes related to xylan degradation, such as one gene for β-1,4-xylanase, one for β-xylosidase, and the other 2 for xylanase/chitin deacetylase were found in the genome [35]. In strain SCUT27, xylanase and β-xylosidase that belong to the glycoside hydrolase family 10 (GH10) have

been cloned and characterized [36,37]. The function of *Tsac_1445* and *Tsac_1464* for xylan/xylose metabolism was also characterized in *T. saccharolyticum* [38]. Additionally, xylose delays cell lysis by sustaining coenzyme and ion metabolism, thus enhancing the viability of the thermophiles [23]. Most of mesophilic strains use glucose preferentially to xylose owing to CCR. However, *Thermoanaerobacter* species can co-utilize xylose and glucose without CCR [22,39] as discussed below.

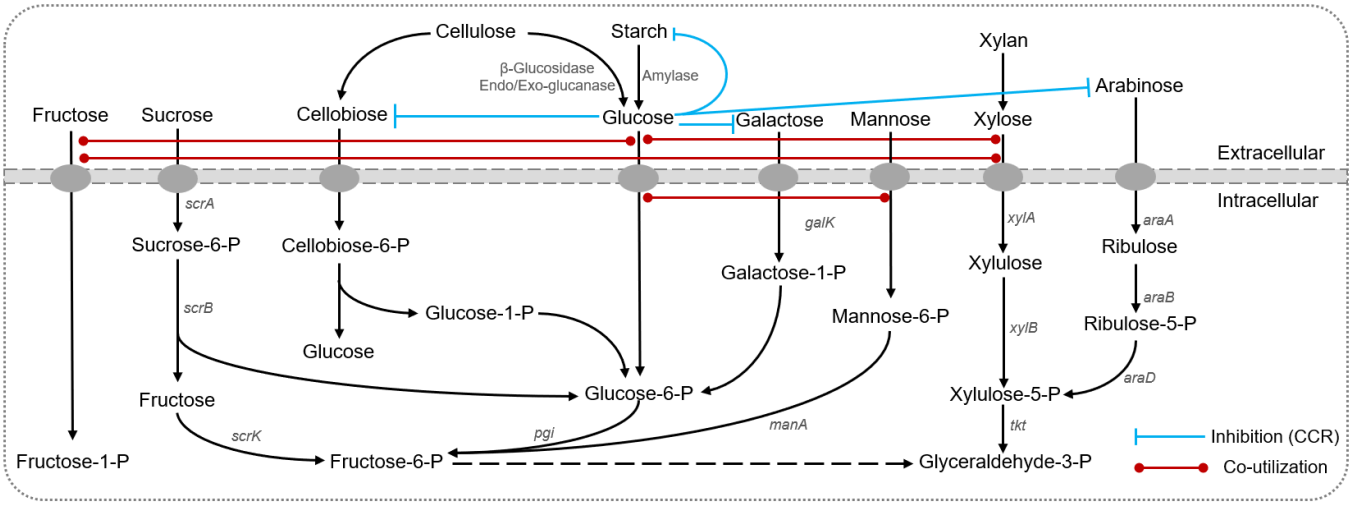

Figure 2. Main sugar metabolism in genus *Thermoanaerobacter*.

2.2. Glucose and Xylose Co-Fermentation in Thermoanaerobacter Species

When cultured with mixed carbon source, microorganisms would selectively use preferred carbon sources by preventing the expression of enzymes participating in secondary substrates [19]. This regulation is called CCR, which benefits microorganisms for competitions in natural environments but prolongs the fermentation periods and increases the costs for industrial applications. Co-fermentation of multiple carbon sources for the production of high value-added biofuels is a promising production strategy. Among the abundant microorganisms in nature, thermophilic anaerobic bacteria such as *Thermoanaerobacter* sp. X514, *T. saccharolyticum* and strain SCUT27 were reported to have the native ability to utilize glucose and xylose simultaneously [22,23,39]. To reveal molecular mechanism of the glucose and xylose co-utilization in *Thermoanaerobacter* sp. X514, the glycobiome was performed and analyzed via two-dimensional transcriptome sequencing and results revealed that the functional modules of glucose and xylose metabolism were independent and regulated by different transcriptional anti-terminators BglGs, which was quite different from xylose transporters regulated by XylR in *Escherichia coli*, *Bacillus subtilis* and *Clostridium tyrobutyricum* [23]. However, the functional modules of glucose and xylose metabolism regulated by BglGs independently in *Thermoanaerobacter* sp. X514 could not perfectly explain the release of CCR between glucose and xylose. It's reported that BglG family transcription antiterminators are key players in the control of carbohydrate catabolism in bacteria [40,41]. These antiterminators are generally composed of three modules, an N-terminal RNA-binding domain (CAT) followed by two homologous regulation modules PRD1 and PRD2 that control the RNA binding activity of the effector via phosphorylation on the conserved histidines [41]. LicT is one member of BglG family transcription antiterminator. The phosphorylation of LicT in PRD1 is catalyzed by P~EIIB^{Bgl} in the absence of β-glucosides and inhibits the activity of LicT. In contrast, when β-glucosides are present, P~EIIB^{Bgl} is preferably transferring the phosphate group to β-glucosides with the PRD1 of LicT unphosphorylated. However, this format of LicT is also non-functional and requires P~His-HPr to phosphorylate PRD2, in the absence of preferred carbon sources such as glucose. When His-100 and His-169 in PRD1 are unphosphorylated, and one or both conserved His in PRD2 are phosphorylated, LicT is activated and binds bound to RNA on the ribonucleic antiterminator to stabilize an RNA secondary structure for the successful transcription as shown in Figure 3. The transcription of operons mediated by BglG is also regulated by the preferred carbon sources. In addition to BglG, both MtlR and LevR, as the transcriptional activators, are also activated by P~His-HPr in the absence of preferred carbon sources [39,42,43], which could explain the catabolite control protein A (CcpA)-independent CCR between glucose and cellobiose in strain SCUT27 [20].

In addition, some other sugars can also be co-utilizaed during fermentation (Figure 2). With the lignocellulosic hydrolysate as substrate, once glucose and xylose concentrations approached zero, mannose and galactose were consumed simultaneously, the concentration of which were decreased by 98% and 92%, respectively. Finally, 37 g/L ethanol was produced from mixed sugars with an average ethanol productivity of 1.5 g/L/h in *T. saccharolyticum* engineered strain (ALK2) [34]. Although strain SCUT27 could co-utilize glucose and xylose, the presence of glucose still repressed xylose catabolism in some extent [44]. Therefore, new microorganism should be obtained by further screening and metabolic engineering. Thermophilic strain like TG57 was selected under the pressure of 2-deoxyglucose with *ccr* and *xylR* deletion/silence and thus was able to simultaneously ferment glucose,

xylose and arabinose to produce butanol (7.33 g/L) as the sole solvent [45]. Other strategies for the enhancing utilization of substrates were also discussed below.

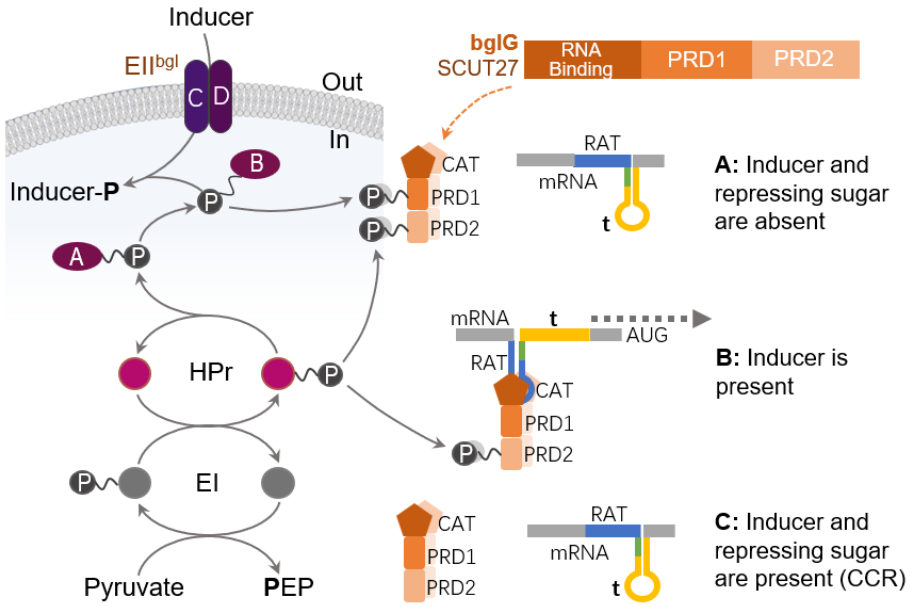

Figure 3. Mechanism of the transcriptional antiterminator BglG regulation [40]. (A) In the absence of the inducer, transcription of PTS-encoding genes is inhibited via the formation of a terminator structure (t, yellow) on the mRNA upstream from the start codon. Under this condition, the antiterminator cannot bind to its RNA target, RAT (blue), because the EIIB is mainly phosphorylated and transfers its phosphoryl group to PRD1 of the antiterminator. The absence of a repressing sugar is also expected to allow phosphorylation at the activating domain (PRD2) by P~His-HPr. However, the negative effect of phosphorylation at PRD1 is dominant. In most antiterminator-controlled PTS operons, the two sites RAT and t overlap (green), and the formation of the terminator therefore prevent the formation of the RAT stem-loop. (B) If only an inducer is present, the EIIB as well as PRD1 of the corresponding antiterminator will be present mainly in an unphosphorylated form. Antiterminator-controlled PTSs are usually the sugar transporters with low-capacity and could be phosphorylated at PRD2 by sufficient P~His-HPr in the absence of repressing sugar. The activated antiterminator binds to RAT and thus favors the formation of the RAT stem-loop (in green), thereby preventing the formation of the terminator. (C) If, in addition to the inducing sugar, a repressing carbohydrate is present, the amount of P~His-HPr will be low in the cells. In firmicutes, P~Ser-HPr will also be formed, which further lowers the amount of P~His-HPr. These conditions prevent the phosphorylation at PRD2, resulting in inactivation of most antiterminators.

2.3. Starch Metabolism in Thermoanaerobacter

Starch is the abundant carbon source in the nature and high-temperature tolerant amylase is urgently needed to degrade the starch for industrial applications. Many types of thermostable enzymes have been studied for decades and one of them is amylopullulanase (APU), which possesses hydrolytic activity towards α-1,4 and α-1,6-glucosidic linkages and has been well-studied for industrial applications [46,47].

Thermoanaerobacter species such as *T. saccharolyticum* and strain SCUT27 can use starch, suggesting that thermostable amylases could be obtained from them [22,38,48]. It's reported that the uitlization of starch was strongly inhibited by 2-deoxyglucose (an analogue of glucose), indicating that the CCR existed between starch and glucose (Figure 2) [22]. The CCR related genes, *ccpA*, *ptsH* and *hprK* were confirmed in strain SCUT27 and the high affinity between CcpA and phosphorylated HPr was proved via affinity chromatography [22]. Currie et al. also studied key hydrolases and transporters (Tsac_1344-1349) employed by *T. saccharolyticum* for starch metabolism, and genes expressions among starch, glucose or xylan were compared, showing a host of differences in terms of transporters, sensory genes, and hydrolases [38]. The highest ethanol titer achieved was 70 g/L in batch culture with a mixture of 90 g/L maltodextrin and 60 g/L cellobiose by *T. saccharolyticum* [49].

2.4. Intracellular Cellobiose Metabolism in Thermoanaerobacter

Cellulose is the major component in most of the lignocelluloses and can be hydrolyzed to glucose by cellulases, including endoglucanase, exoglucanase and β-glucosidase. Cellobiose is abundant in partial hydrolysis of lignocellulosic hydrolysates due to the weak activity of β-glucosidase, and the rate-limiting enzyme in the enzymatic hydrolysis [50]. There are three types of industrial hosts with the capability to use cellulose: (1) uptaking glucose from cellulose with cellulase pretreatment; (2) utilizing cellobiose extracellularly from partial hydrolysis of cellulose with the ability to produce β-glucosidases; (3) assimilating cellobiose intracellularly from partial cellulose hydrolysis with transporters and intracellular β-glucosidases [51]. Several critical barriers existed with the complete extracellular hydrolysis of cellulose, such as high costs for the supplementation of β-glucosidase, CCR caused by the released glucose during hydrolysis and energy waste for glucose metabolism instead of cellobiose [17,52].

The genus *Thermoanaerobacter* was engineered to overcome these obstacles for intracellular assimilation of cellobiose. Like strain SCUT27, it can assimilate cellobiose intracellularly with CCR existing between cellobiose and glucose [20,22]. Cel2 was the only intact operon for cellobiose utilization including integral PTS transport elements and glycoside hydrolase, the transcription of which was regulated by σ^{54}-dependent transcription activator $CelR^{cel2}$. By replacing the σ^{54}-dependent promoter of Cel2 with the strong promoter from gene *adhE*, the mutant obtained the ability to co-utilize glucose and cellobiose [20]. In hemicellulose-degrading *T. saccharolyticum***,** the biochemical and structure of a glucose-tolerant β-glucosidase was analyzed by Kim et al and results showed that TsaBgl had the unique conformation compared with other structurally known Bgls, and it also could convert cellobiose as well as other soluble cello-oligomers into glucose, providing energy sources for living organisms [53]. In *T. saccharolyticum*, 41 g/L ethanol was achieved from enzymatic hydrolysate of hardwood containing 96 g/L mixture of glucose and cellobiose [54] and 70 g/L ethanol was obtained in batch fermentation with a mixture of cellobiose and maltodextrin [49].

2.5. Traits about Metabolism of Other Substrates

In addition to the characteristics of sugar metabolism mentioned above, there are some other traits about sugar metabolism in the genus *Thermoanaerobacter*. In *T. saccharolyticum*, it was found that the utilization of mannose and arabinose was started before glucose and xylose were exhausted, owing to some thermostable enzymes efficient in *Thermoanaerobater*, such as β-mannanase [34,55]. However, in strain SCUT27, the metabolism of galactose and arabinose was inhibited by glucose, while mannose could be co-fermented with glucose [22]. In *T. saccharolyticum*, arabinose was completely utilized even at high concentrations, the metabolism of which was suppressed significantly at the presence of glucose or mannose. Directed mutations of *hpr* resulted in a reversal of carbon source preferences, and the mutant (His15Asp) utilized arabinose first, then followed by glucose, slowly and incompletely [54]. Notably, in *T. saccharolyticum*, CCR of galactose, arabinose and cellobiose in the presence of 2-deoxyglucose could be relieved by the HPr (His15Asp) mutation [39].

The central carbon metabolic network of *T. saccharolyticum* was established based on isotopic tracing and results showed that the metabolism of glucose was exclusively depended on EMP pathway, and both the Entner-Doudoroff (ED) pathway and the oxidative pentose phosphate pathway (oxPPP) were inactive [56]. Additionally, some thermophiles such as *T. pseudethanolicus* 39E and *T. thermosaccharolyticum* are capable of metabolizing sucrose [33]. Interestingly, *T. butyriciformans* sp. nov. USBA-019^T and *T. thermosaccharolyticum* MJ1, presented different products composition under various substrates [57,58]. The main fermentation products of *T. butyriciformans* sp. nov. USBA-019^T under glucose, lactose, cellobiose, galactose, arabinose, xylose, starch and xylan were acetate and butyrate. Other products, such as ethanol and lactic acid, also could be produced under starch and cellobiose [57]. Mannitol and glycerol were the more reduced substrates than glucose, and *T. mathranii* could use mannitol and glycerol to acquire higher ethanol yield [59]. Recently, it was reported that *T. kivui* as an acetogenic model organism could reduce CO_2 with electrons derived from H_2 or CO, or from organic substrates in the Wood–Ljugdahl pathway (WLP) [60,61], further broadening the substrate spectrum of *Thermoanaerobater*.

3. Genetic Operation Platforms

3.1. Vectors for Gene Overexpression

This section would provide a comprehensive description about the current genetic tools and techniques for modification of the genus *Thermoanaerobacter*, which belongs to low-G+C thermophilic and obligate anaerobes in the class *Clostridia*, and is considered to be resistant to genetic engineering due to the difficulty of introducing foreign DNA to cells. Shaw et al. reported the evidence of natural genetic competence in 13 kinds of *Thermoanaerobacter* and *Thermoanaerobacterium* strains, which were previously believed to be difficult to be transformed or genetically recalcitrant [62]. And in *T. saccharolyticum* JW/SL-YS485, natural competence-mediated DNA incorporation occurs during the exponential growth phase with both replicating plasmid and circular or linear DNA [62]. The transformation protocol and methods for transformants selection of *T. saccharolyticum* are described by Hon et al [48].

The earliest and most common vector for transformation and gene-overexpression in the genus *Thermoanaerobacter* such as *Thermoanaerobacterium* sp. strain JW/SL-YS485 was the plasmid pIKM1 containing kanamycin resistance gene (resistance gene of high-temperature kanamycin, *htk*) [54,63]. This replicating plasmid was originated from a 2.4 kb thermophilic replicating plasmid in *T. saccharolyticum* strain B6A-RI with KanR, AmpR, and a pUC origin, and then developed to create a convenient cloning vector [54]. Additionally, pTE16 was constructed based on the *E. coli-Clostridium perfringens* shuttle vector pJIR715 with a thermostable chloramphenicol (Cm) resistance cassette. The electro-transformation of *T. ethanolicus* JW200 was performed with pTE16 and the transformation efficiency was 50 ± 7.4 transformants per μg plasmid DNA [64]. Based on the hyperthermophilic *Caldicellulosiruptor bescii* replicon pBAS2, Joseph et al. constructed vectors which could be transformed into some thermophiles at 60 °C and maintained in multiple copy with the replication initiation protein RepL [65]. Le et al. successfully constructed an *E. coli-T. ethanolicus* shuttle expression vector pBlu10-*htk* for effective heterologous expression [66]. Some heterologous genes expression gave the strains expected performances successfully. For example, a heterologous gene *gldA* encoding an NAD^+-

dependent glycerol dehydrogenase was expressed to facilitate NADH regeneration for enhancing ethanol formation in *T. mathranii* [59]. Genes encoding the urease were integrated in *T. saccharolyticum* to hydrolyze urea [67].

3.2. Gene Knockout by Homologous Recombination

Kanamycin, erythromycin, chloramphenicol and thiamphenicol are four common antibiotics that can be used for the positive selection of the transformants from the genus *Thermoanaerobacter* (Table 1) [34,64]. Kanamycin and thiamphenicol are generally preferred due to the lower background and high efficiency of transformation. Notably, thiamphenicol or chloramphenicol is not used for *T. thermosaccharolyticum*, which carries acyltransferase gene like thiolase (*thl*). And these antibiotics can work in conditions that are suitable for the optimal growth of the genus *Thermoanaerobacter* (i.e., temperature and pH) under the responding working concentration as shown in Table 1 [34,64,68]. Haloacetates, 5-fluoroorotic acid (5-FOA) and 5-fluoro-2'-deoxyuridine (5-FUDR) are the negative selective drugs and can be adopted for the selection of transformants from genus *Thermoanaerobacter* with the corresponding endogenous negative selection markers genes, *pta-ack*, *pryF* and *tdk* deleted, respectively [48,69]. Several kinds of editing ways such as deletions, insertions, or a combination of a deletion and an insertion by homologous recombination in the genome could be achieved by the transformation of the suicide plasmid (circular DNA) like pBluescript II SK(+) or PCR products (linear DNA). Markerless gene deletion strategies were developed based on the counter-selectable markers, such as *pyrF*, *tdk* and *pta*/*ack* genes, which could eliminate the constraints imposed by the small number of positive selection markers for *T. saccharolyticum* [70]. The design of the suicide vector for markerless editing was also described in several studies [48,69,70].

Table 1. List of the practicable selection marker genes and the responding agents for *Thermoanaerobacter* species genetically engineering.

Selection Marker Gene	Selective Drugs	Solvent	Working Concentration (μg/mL)	Selection Type	Ref.
kan (*htk*)	Kanamycin	water	50–200	Positive	[63,71]
erm	Erythromycin	2 M HCl or ethanol	5–10	Positive	[34,68]
cat	Chloramphenicol or Thiamphenicol	water	40–80; 5–10	Positive	[64,65]
pyrF(*pyrE*)	5-fluoroorotic acid (5-FOA)	Dimethyl sulfoxide	100	Negative	[70,72]
pta-ack	Haloacetates (etc. Sodium chloroacetate)	water	23.3	Negative	[70]
tdk	5-fluoro-20-deoxyuridine (FUDR)	water	10–20	Negative	[73]

3.3. Application of CRISPR/Cas Genome Editing System in Thermophiles

Recently, there are some application of Clustered Regularly-Interspaced Short Palindromic Repeat (CRISPR)/cas (CRISPR associated) system in thermophiles. Compared with traditional makerless editing, the advantage of the CRISPR system is to make the markerless editing easier for just one step. Mougiakos et al. identified and characterized ThermoCas9 from the thermophilic bacterium *Geobacillus thermodenitrificans* T12, which was active between 20 and 70 °C *in vitro* [74]. And thermoCas9-based engineering tools for gene editing such as gene deletion and transcriptional silencing at 55 °C in *Bacillus smithii* were performed successfully [75]. Le et al. developed a thermostable Cas9-based system for genome editing of *T. ethanolicus*, in which three genes (*tdk*, *adhE* and *rsp*) were edited successfully [66].

However, the heterologous Cas9 generally was poisonous to the cells [76,77]. Therefore, the native CRISPR/Cas system of some thermophiles was developed. For *Thermoanaerobacter* species, Dai et al. developed the endogenous type I-B CRISPR/Cas system with thymidine kinase gene (*tdk*) as a negative selection marker for multiplex genome editing in strain SCUT27, and the engineered strain SCUT27/Δ*tdk*/Δ*ldh*/Δ*argR* exhibited the significant improvement over the wild type on ethanol production and xylose utilization [76]. In *C. thermocellum*, Walker et al. also repurposed the native Type I–B CRISPR/cas systems for genome editing [78]. Additionally, three thermophilic Cas9 variants (heterologous Type II) were tested and results showed that only GeoCas9, isolated from *Geobacillus stearothermophilus*, was active in *C. thermocellum* [78]. To overcome the limitation of homologous recombination between the repair template and the genome for both the Type I–B and the Type II GeoCas9 system, three novel thermophilic recombinases were tested, and only the exo/beta homologs, isolated from *Acidithiobacillus caldus*, exhibited the activity in *C. thermocellum* and led to higher genome editing efficiency [78].

The CRISPR-based transcriptional regulation of repression and activation (CRISRP*i* & CRISRP*a*) is a simple and high-throughput genome engineering tool and has been successfully performed in the genus *Clostridium* but rarely in thermophiles [79–82]. Nowadays, Ganguly et al. developed a ThermoCas9-based CRISPR interference (CRISPR*i*) system, which was applied to repress the transcription of lactate dehydrogenase (LDH) and phosphotransacetylase (PTA) genes in *T. thermocellum* with 67% and 62% enzymatic activity loss, respectively, without gene damage [83]. In addition to the heterologous Type II CRISPR*i* system, native Type I system was developed in *Haloferax volcanii*, *E. coli*, *Listeria monocytogenes* and *S. thermophilus* as well as in plants and even human cells [84–86], implying the potential application of endogenous type I CRISRP*i* or CRISRP*a* system in the genus *Thermoanaerobacter*.

4. Strategies for Enhancing the Biofuel Production

4.1. Enhancement of Substrates Utilization

The performance of the utilization of various substrates in *Thermoanaerobacter* has been reviewed above (section 2). However, there are not so many researches about the localization and class of each sugar transporters in *Thermoanaerobacter*, as well as the responding modification. To improve the tolerance to substrates, the strain Δ*ldh*/Δ*pta-ack* of *T. saccharolyticum* was continuously cultured for approximately 3000 h with progressively increased feed xylose concentrations and finally the mutant exhibited a greater capacity for xylose consumption. More than 99% of the feed xylose was metabolized at concentrations up to 70 g/L with a mean ethanol yield of 0.46 g/g in continuous culture at pH of 5.2–5.4 without base addition [34]. In addition to the adaption evolution under high concentration substrates, genetic modification is also a key strategy to improve substrate utilization. Four enzymes from *Herbinix spp.* strain LL1355 which could hydrolyze almost all of glucuronoarabinoxylan linkages were successfully expressed in *T. thermosaccharolyticum*, allowing it to consume more substrates than that of the parent strain (78% vs. 53%) with ethanol yield improved by 24% from corn fiber [87]. Arginine repressors (ArgRs), the transcriptional repressors of the arginine biosynthesis, were reported to be involved in several key metabolic pathways and might be the global regulator [88,89]. In strain SCUT27, the deficiency of *argR* (V518_1864) could greatly improve the co-utilization of glucose and xylose, due to the enhanced activity of xylose isomerase, xylulokinase and the higher energy level [44]. Deletion of the redox-sensing transcriptional repressor (Rex) also improved the ability of sugar consumption of SCUT27 about 74–131% with the improvement of total ATP concentration and the cofactors NADH and NAD^+ concentrations [90].

Consequently, further work can be carried out to improve the substrates utilization from the following three aspects: (i) Figure out and strengthen the transporters of sugars in *Thermoanaerobacter*; (ii) Understand the components and effectors of CCR in *Thermoanaerobacter*, and realize the co-utilization among the sugars and even expand the substrate spectra; (iii) High substrate tolerant strain should be screened or engineered by adaptive evolution and random mutation.

4.2. Enforcement of the Metabolic Flux to Biofuels Production

Biofuels are emerged as a promising alternative to fossil fuels for meeting the rapid increases of energy consumption and accumulation of greenhouse gases (climate change) [14]. For the genus *Thermoanaerobacter*, there are mainly three biofuels products: ethanol, hydrogen and butanol. Ethanol and hydrogen are principal products for majority of the *Thermoanaerobacter* species while butanol is only produced in a part of the genus *Thermoanaerobacter* like *T. thermosaccharolyticum* [91], which has the metabolic pathways of butyrate and butanol (Figure 4). In addition to biofuels, other bioproducts like lactic acid and acetic acid were rarely reported to be produced by *Thermoanaerobacter* species [60,61,92]. So, here we mainly discuss the research of *Thermoanaerobacter* species on biofuels production.

4.2.1. Ethanol Production

Bioethanol is one of the most attractive biofuels as it can serve as a blendstock for gasoline [93]. As a result, several countries, including the USA, Brazil, China, Canada, India and many EU members, have already proclaimed commitments to reduce their dependence on fossil fuels towards developing bioethanol production [94]. Currently, bioethanol is commonly produced by *Saccharomyces cerevisiae* [95,96], *Zymomonas mobilis* [97,98] and *E. coli* [96,99]. *Thermoanaerobacter* species are good ethanol producers at 55 °C (the temperature more economical to recover ethanol by continuous distillation) [54,100,101]. Strategies for enhancing the ethanol production mainly include strengthening the pathway of ethanol production, lowering the pathway of by-products, regulating the intracellular cofactor regeneration system as well as ATP level, or adaptive evolution (Table 2).

Both pyruvate formate lyase (PFL) and pyruvate ferredoxin oxidoreductase (PFOR) were found to participate in acetyl-CoA formation from pyruvate in genus *Thermoanaerobacter* [102]. PFOR is the key enzyme for high ethanol production in genus *Thermoanaerobacter*, because it is not inhibited by accumulation of ethanol or NADH while PFOR of *C. thermocellum* is [103]. Acetyl-CoA would be catalyzed by three major *adh* genes (*adhA*, *adhB*, and *adhE*) for ethanol production [33]. In addition, a few thermophilic bacterial species within the genus *Thermoanaerobacter* harbored a copy of aldehyde:ferredoxin oxidoreductase (*aor*), which reduced a variety of organic acids to their corresponding aldehydes, showing the AOR-ADH pathway in strains like *Thermoanaerobacter* sp. strain X514, *T. brockii* ssp. *Finnii* and *T. pseudethanolicus* [104–106]. AdhE is the bifunctional enzyme including alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities, the deletion of which would reduce the ethanol yield by ~95% [107]. NADH was the preferred cofactor for both ALDH and ADH activities [108]. It was reported that deletion of *adhE* reduced NADH-dependent ADH activity by 98%, which did not affect the NADPH-dependent ADH activity [72]. Notably, some mutants with high ethanol production, showed the increased NADPH-dependent ALDH and ADH activities, in which deletion of *adhA* dramatically reduced NADPH-dependent ADH activity and ethanol production [72,108]. Moreover, multiple gene deletions of all annotated alcohol dehydrogenase genes showed that AdhA and AdhE were the two main alcohol dehydrogenases involved in ethanol production in *Thermoanaerobacter* specie [72]. Thus, AdhA is responsible for the NADPH-ADH activity in *Thermoanaerobacterium* specie, especially in high-ethanol-producing strains, and may also serve as an additional

acetaldehyde scavenger [72]. However, the overexpression of bifunctional or monofunctional ADH genes in wild type or engineered strains seems no improvement in ethanol titer [54].

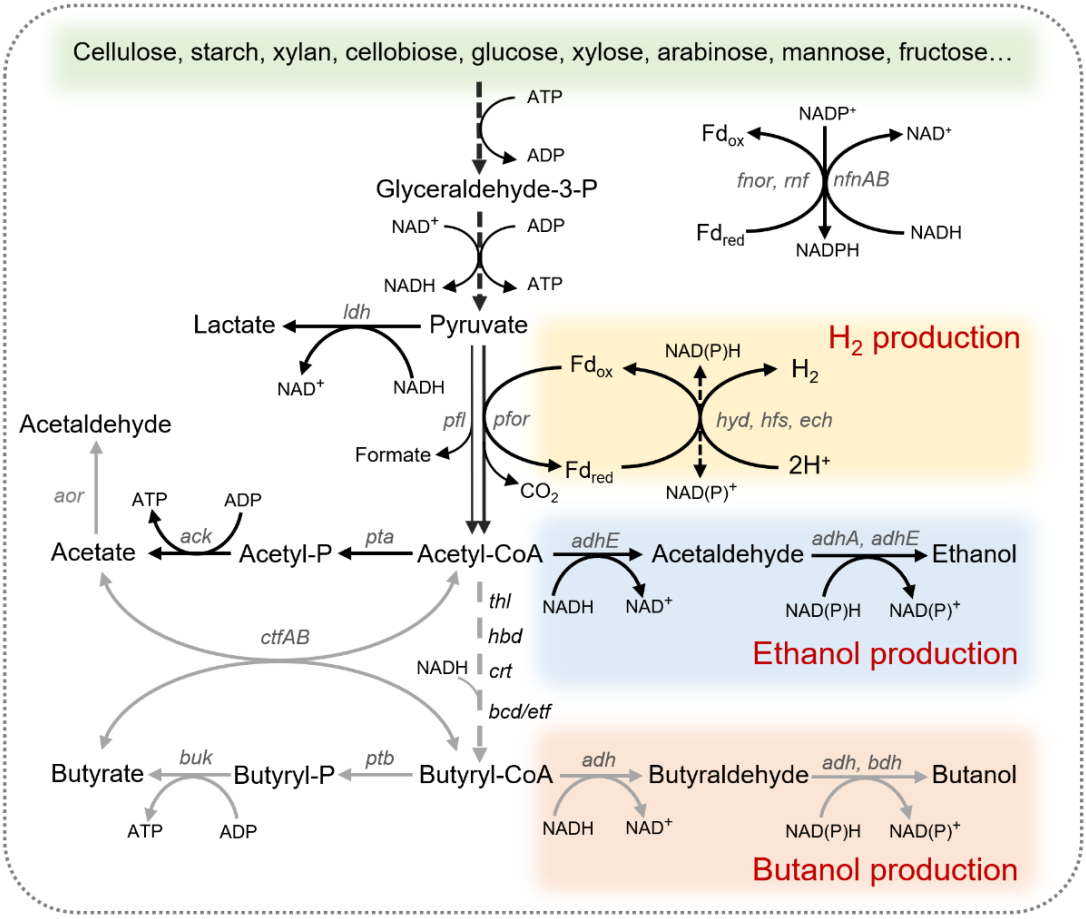

Figure 4. The metabolic pathway for H_2, ethanol and butanol synthesis in *Thermoanaerobacter* species. The H_2 and ethanol formation pathway in *Thermoanaerobacterium* species while the butanol formation pathway in *T. thermosaccharolyticum* only. *ldh*, lactate dehydrogenase; Fd_{ox}, pyruvate:ferredoxin oxidoreductase oxidized; Fd_{red}, pyruvate:ferredoxin oxidoreductase reduced; *hyd*, *hfs* and *ech*, three hydrogenases; *pta*, phosphotrans acetylase; *ack*, acetate kinase; *adh* (*adhE*, *adhhA*), alcohol/aldehyde dehydrogenase; *bdh*, butanol dehydrogenase; *buk*, butyrate kinase; *ptb*, butyryl-CoA dehydrogenase; *ctfAB*, CoA transferase; *thl*, thiolase; *hbd*, β-hydroxybutyryl-CoA dehydrogenase; *crt*, crotonase; *bcd*, butyryl-CoA dehydrogenase; *etf*, electron transferring flavoprotein; *nfnAB*, NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase; *pfl*, pyruvate formate lyase; *pfor*, pyruvate ferredoxin oxidoreductase. *aor*, aldehyde:ferredoxin oxidoreductase. The grey arrows show the pathways only existed in a part of strains.

For higher ethanol production by redistribution of carbon flow, metabolic engineering of the genus *Thermoanaerobacter* began with the disruption of the lactate production pathway [100,109]. Compared with the wild type, the biomass of mutant Δ*ldh* without lactic acid production, was increased by 31.0–31.4% under glucose or xylose as well as 2.1 to 2.4-fold increases in the yield of ethanol (mole/mole substrate) [100]. Shaw et al. knocked out the genes involved in organic acid formation (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase) and the resultant strain was able to produce ethanol as the sole detectable product (90% of the theoretical maximum yield) and 37 g/L ethanol was produced from mixed sugars with a maximum ethanol productivity of 2.7 g/L/h [34]. Shaw et al. investigated three putative hydrogenase enzyme systems in *T. saccharolyticum* and found that the strain with specified *hfs* and *ldh* deletions exhibited an increased ethanol yield from consumed carbohydrates [110,111]. The deletion of *hfsB* was also performed in strain SCUT27 and results indicated the enhanced ethanol production was obtained, due to the increased NADH supply and alcohol dehydrogenase activity [112]. Pyruvate formate lyase-activating protein (PflA) could assist the formation of pyruvate formate lyase (Pfl), which catalyzed pyruvate into acetyl-CoA and formate. The formation of formate also affected the ethanol production by changing the reducing power supply [101]. In strain SCUT27, the mutant Δ*ldh*/Δ*pflA* was constructed and it could produce 45 g/L ethanol during immobilized cell fermentation, owing to the increased electrons available for ethanol production [101]. The enhanced ethanol/acetate ratio was also important for ethanol production, which contributed to the resistant to 5% (*v*/*v*) exogenous ethanol [101]. To decrease amino acid secretion and reduce ammonium assimilation in *C. thermocellum*, type I glutamine synthetase gene (*glnA*) was knocked out and the levels of secreted valine and total amino acids were reduced by 53% and 44%, respectively, along with ethanol yields increased by 53% [27].

Additionally, reducing equivalents are key factors of alcohol production in *Thermoanaerobacter* species. There are three types of ferredoxin:NAD(P)H oxidoreductase (FNOR) reactions: uncoupled FNOR reaction, proton- or Na^+-translocating FNOR reaction (RNF) and NADH-dependent FNOR reaction (NFN) [113]. NADH-FNOR (Tsac_1705) which transfers electrons from the reduced ferredoxin to NAD^+, is the main enzyme responsible for ferredoxin oxidization in the NADH-based ethanol pathway in *T. saccharolyticum* [113]. In the high-ethanol-producing mutant (Δ*ack*/*pta*), NADPH-FNOR activity was increased compared with that of the wild type, which presumably led to the increased NADPH production [72,114]. The electron-bifurcating enzyme complex NfnAB (one of NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductases, NFN), which transfers electrons from the reduced ferredoxin and NADH to 2 $NADP^+$, is thought to play a key role in linking NAD(P)(H) metabolism with ferredoxin metabolism and has been hypothesized to be the main enzyme for ferredoxin oxidization in mutants of *T. saccharolyticum* for increasing ethanol production [114]. In strain SCUT27, the NADH/NAD^+ ratio in the Δ*nfnAB* mutant was increased [68]. Interestingly, *adhA* is directly located in the upstream *nfnAB* in *T. saccharolyticum*, showing the potential link between reducing power supply [114]. Two different mechanisms for stoichiometric yield of ethanol were proposed in *T. saccharolyticum*. One was based on NADPH and NfnAB, and the other was dependent on NADH and a yet undescribed NADH-FNOR (like unknown *rnf* complexes) [114]. The redox-sensing transcriptional repressor Rex, which was widely reported as the targeting *adhE* by sensing the redox state, was knocked out in strain SCUT27. Results showed that the ethanol yield of strain SCUT27/Δ*rex* was increased by 75–91% within different carbon sources, along with the improvement of total ATP, cofactors concentration as well as the activities of alcohol dehydrogenase [90]. To regenerate cofactor, *T. mathranii* was fed with mannitol to increase ethanol yield [59]. To further facilitate NADH regeneration for ethanol formation, a heterologous gene *gldA* encoding a NAD^+-dependent glycerol dehydrogenase was overexpressed in *T. mathranii*, and the resultant mutant showed increased the ethanol yield in the presence of glycerol undr xylose [59]. However, it was also reported that a wide range of genetic modifications for the mixed acid fermentation pathways did not significantly alter the NAD(P)H/$NAD(P)^+$ ratios as well as their concentrations in *T. saccharolyticum* [115]. It appeared that for each species, the ratios were maintained in a somewhat narrow range: 0.4–0.7 (NADH/NAD^+) and 0.5–0.9 (NADPH/$NADP^+$) [115].

Moreover, adaptive evolution under exogenous ethanol did not always result in mutants of *T. saccharolyticum* that could produce more ethanol and in fact, the mutant obtained usually produced lower titer of ethanol than the wild type [54]. A strain of *T. pseudethanolicus* with enhanced ethanol tolerance showed high yields of ethanol but lower titer than the parent strain, and NADH-dependent ADH, ALDH, and FNOR activities of the mutant were lost, but NADPH-dependent activities remained [116]. Interestingly, addition of acetate showed an unexpected stimulatory effect on ethanolic fermentation by *T. ethanolicus* strain 39E and strain X514 while lactate was in general inhibitory to ethanolic fermentation. In *T. saccharolyticum* M2886, the highest ethanol titer achieved was 70 g/L (also the maximun ethanol tolerance) in batch culture under the mixture of cellobiose and maltodextrin by removing exopolysaccharide synthesis gene (*perR*) and replacing the gene of methyglyoxal synthase with *pta/ack* genes, after multiple rounds of adaptation and selection [49]. Furthermore, it suggested that vitamin B12 de novo synthesis in strain X514 had a significant positve effect on ethanol yields in both mono- and coculture [33]. The vitamin B12 pathway may appear to promote ethanologenesis through ethanolamine and 1,2-propanediol [23]. Additionally, conversion of acetic acid to acetone in engineered *T. saccharolyticum* resulted in the improved ethanol productivity and titer, and was an attractive low-cost solution to acetic acid inhibition [117].

To sum up, ethanol production in *Thermoanaerobacter* species could be further improved by conducting more carbon flux to ethanol and weakening the reducing power consumed by the branch-pathways for enhancing ethanol production. In addition, high-ethanol-tolerant strain should be screened by adaptive evolution or random mutation. Finally, future studies on ethanol production would focus on the cheaper and less toxic feedstocks to improve their application value.

4.2.2. H_2 Production

Hydrogen produced from biomass feedstocks is considered as an effective solution toward a decarbonized economy [118]. Through the circular economy framework, it is possible to produce the sustainable biohydrogen and other bioproducts while address the issues such as waste management [119]. In *Thermoanaerobacter* species, hydrogen is a necessary product for the disposal of the reducing equivalents. Strains like *T. tengcongensis* are able to convert the reducing equivalents generated during the fermentation from glucose to the acetate and CO_2 by reducing H^+ to H_2 (Table 3). A unique combination of hydrogenases, a ferredoxin-dependent [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase, was found to be responsible for H_2 formation in *T. tengcongensis* [120].

Table 2. Summary of the ethanol production by *Thermoanaerobacter* species and common ethanologenic strains.

Strain	Genotype	Substrate	Substrate Concentration (g/L)	Fermentation Mode	Ethanol Concentration (g/L)	Ethanol Yield (g/g)	Productivity (g/L/h)	Ref.
T. aotearoense SCUT27	Wild type	Glucose/xylose	9.0/8.6	Batch	1.5/1.5	0.16/0.17	/	[68]
T. aotearoense SCUT27/*Δldh*	*Δldh*	Glucose	105.5	Fed-batch in FBB	35.6	0.34	/	[101]
T. aotearoense SCUT27/*Δldh* /Δ*pflA*	Δ*ldh*, Δ*pflA*	Glucose	127	Fed-batch in FBB	45	0.36	/	[101]
T. aotearoense SCUT27/*Δldh* /Δ*hfsB*	Δ*ldh*, Δ*hfsB*	Glucose	111	Fed-batch in FBB	39.6	0.36	0.41	[112]
T.aotearoense SCUT27/Δ*argR1864*	Δ*argR*	Rice straw, Corn cob, Sugarcane Bagasse	~15	Batch	5.2–5.6	0.35–0.40	/	[44]
T.aotearoense SCUT27/*Δrex*	Δ*rex*	Glucose	~25 g/L	Batch	4.56	0.32	/	[90]
T.aotearoense SCUT27/*Δrex*	Δ*ldh, Δrex*	Glucose	~25 g/L	Batch	7.45	0.38	/	[90]
T. saccharolyticum YS485	Wild type	Cellobiose	4.6	Batch	1.2	0.24		[110]
T. saccharolyticum ALK2	Δ*ldh*, Δ*ack*, Δ*pta* and adaptive evolution	Xylose	70	Continuous	33	0.46	2.2	[34]
		Sugar mixtures	50	Fed-batch	37	-	2.7	
T. saccharolyticum M1442	*Δldh, Δack, Δpta, ΔTsac_0795, ΔEPSoperon, urease* (+)*, metE* (+)	Cellobiose + Maltodextrin ($CaCO_3$)	60 + 90	Batch	70.1	0.47	/	[49]
		Mock hydrolysate	/	SSF	61	0.47	2.13	
		Pre-treated hardwood	/	SSF	31	/	/	
T. saccharolyticum M1051	Δ*ldh* Δ*pta* Δ*ack* and expression of *ureABCDEFG*	Lignocellulose (Urea)	/	/	54.3	/	/	[67]
E. coli	*ΔldhA, Δack, ΔpflB,*	Xylose, Xylose + glucose	50 25 + 25	Batch	25.0 24.7	0.49 0.43	0.3 0.7	[99]
Z. mobilis	Overexpression of *xylA, xylB, tal,* and *tktA*	Acid hydrolysate of wood biomass	~90	Batch	35.1	/	/	[121]
S. cerevisiae	Expression of *mhpF*	Glucose	150	Batch	74.5	97.4%	/	[96]

Three putative hydrogenase systems were identified in *T. saccharolyticum*: A four-gene operon containing two [FeFe]-hydrogenase genes (*hfsBD*), provisionally termed *hfs* (hydrogenase-Fe-S), of which the deletion resulted in a growth defect [111], was found to be the main enzymatic catalyst of hydrogen production; A second hydrogenase gene cluster, a bifurcating NAD(P)H-linked [FeFe]-hydrogenase (*hyd*), exhibited methyl viologen-linked hydrogenase enzymatic activity and was specific to the transfer of electrons from NAD(P)H to H^+; A third gene cluster, a putative [NiFe]-hydrogenase with homology to the *ech* genes, did not exhibit the hydrogenase activity [110,112]. Strains with a deletion of the *hfs* genes exhibited a 95% decrease in both hydrogen and acetic acid production, and *hfsB* seemed as the most important gene of the operon *hfsABCD* [110]. The genes *hyd*, *ech* and *hfsABCD*, highly homologic to that of *T. saccharolyticum* and *T. tengcongensis*, were also found in strain SCUT27 and proved to be related to H_2 production by gene knockout [112].

To redirect the NADH flow to hydrogen production, the gene encoding L-lactate dehydrogenase was knocked out, resulting in 2 and 2.5-folds increase of H_2 yield and production rate, respectively [69]. In the wild type of *T. saccharolyticum*, deletion of *nfnAB* showed a 46% increase in H_2 formation owing to the enhanced NADH generatation [114]. Also, in strain SCUT27, the concentration of hydrogen production was increased by 41.1% due to the enhanced NADH/NAD^+ ratio in the Δ*nfnAB* mutant [68]. Additionally, deletion of PFL, potential H_2-uptake (NAD(P)H-dependent) H_2ases and ethanol producing pathways and overexpression of Fd-dependent H_2ases could also increase H_2 yields and production [122]. However, when H_2 was accumulated in the closed system, the fermentation was partially shifted to ethanol production, due to the high hydrogen partial pressure [$p(H_2)$] leading to the low NADH-dependent hydrogenase activity [121].

Furthermore, a number of H_2-producing *Thermoanaerobacter* species were screened and co-cultured with other strains for higher H_2 production. Hu et al. isolated a new *T. thermosaccharolyticum* named MJ1 from paper sludge, whose hydrogen yields could reach 11.18 mol-H_2/mol cellobiose at an initial sugar concentration of 5 g/L, and the hydrogen production was 111.8 mM under sugarcane bagasse hydrolysate pretreated with 60% dilute-acid (1%, *g*/*v*) [58]. Li & Zhu took a biphasic fermentation approach for the production of ethanol and hydrogen from cassava pulp. The glucose generated by co-culturing of *C. thermocellum* and strain SCUT27 under cassava pulp was utilized rapidly by subsequently inoculated *S. cerevisiae*, and the final productions of 8.8 g/L ethanol and 4.1 mmol/L hydrogen were achieved [123]. *C. thermocellum* and strain SCUT27 were also co-cultured with 40 g/L alkali-pretreated sugarcane bagasse (SCB) for bio-hydrogen production, with a final hydrogen production of ~50 mmol/L [124].

For enhanced thermophilic biohydrogen production with cellulosic wastes, future work can be carried out from the following aspects: First, digging out new thermophilic strains which can naturally produce high level of hydrogen efficiently. Second, providing new strategies for genetic modifications on *Thermoanaerobacter* species for higher hydrogen production, such as developing favorable thermophilic biohydrogen pathways and strengthening enzymes stability. Third, co-cultureing with other microorganisms for further improving the substrates utilization or product yields. Finally, enhancing the biohydrogen production technology under the dark fermentation mode with the implementation of agroindustrial wastes as potential substrates, and exploring the suitable temperature and pH which are the imperative parameters during the dark fermentation.

4.2.3. Butanol Production

Except for the general products of hydrogen and ethanol, some *Thermoanaerobacter* species like *T. thermosaccharolyticum* also have great potential to produce butanol (Table 4), which is a renewable alternative to gasoline in vehicles without any modifications in engine system [2] due to its higher boiling point [125].

A novel thermophilic and butanogenic *T. thermosaccharolyticum* M5 was isolated and could directly produce butanol from hemicellulose via a unique ethanol–butanol (EB) pathway, with the efficient expression of xylanase, β-xylosidase and the bifunctional alcohol/aldehyde dehydrogenase (AdhE) [91]. In addition, process optimization based on the characteristic of AdhE was carried out, which further improved the final butanol titer to 1.17 g/L from xylan [126]. Furthermore, the co-culture of *Thermoanaerobacterium* sp. M5 and *C. acetobutylicum* NJ4 was established to improve the butanol titer to 8.34 g/L from xylan through CBP [126]. Moreover, Li et al. reported a wild-type *T. thermosaccharolyticum* strain TG57, which was capable of converting microcrystalline cellulose directly to butanol (1.93 g/liter) as the only final product. And the strain TG57 was also able to simultaneously ferment glucose, xylose, and arabinose to produce 7.33 g/L butanol [45]. Integration of heterologous components of n-butanol pathway (*thl-hbd-crt-bcd-etfAB*) from *T. thermosaccharolyticum* DSM 571 resulted in 0.85 g/L butanol from 10 g/L xylose in *T. saccharolyticum* JW/SL-YS485 (21% of the theoretical maximum yield), and resulted in 1.05 g/L butanol from 10 g/L xylose in lactate deficient strain (26% of the theoretical maximum yield) [127].

In general, the titer of butanol produced by metabolic engineering strategies needs to be improved due to the high toxicity of butanol and the low catalytic activity of enzymes. The following works can be adopted to optimize the butanol biosynthesis: (i) eliminate the byproduct like ethanol to improve the yield of butanol; (ii) obtain the efficient and thermotolerant enzymes by rational engineering or direct evolution for butanol producing; (iii) improve cell tolerance to butanol via transporter engineering or evolution engineering; (iv) screen novel thermophilic butanol producers that could produce much higher butanol titer than that of *C. acetobutylicum*.

Table 3. Performance comparison of *Thermoanaerobacter* species for biohydrogen production.

Organism	Genotype	Carbon Sourse	Substrate Concentration (g/L)	H_2 Production (mmol/L)	Yield (mol/mol)	Productivity [mmol/(L·h)]	Ref.
Thermoanaerobacterium sp. strain F6	Wild type	Xylan	60	370.7	/	/	[30]
Thermoanaerobacterium sp. strain F6	Wild type	Corn cob	/	66.7	/	/	[30]
T. aotearoense SCUT27	Δ*ldh*	Glucose, xylose	15	/	1.5–2.7	10.3–20.9	[68]
T. aotearoense SCUT27/Δ*nfnAB*	Δ*nfnAB*	Glucose, xylose	10	44.8–49.3	1.4–1.7	/	[71]
T. aotearoense SCUT27/Δ*nfnAB*	Δ*nfnAB*	Rice straw and corn cob hydrolysates	/	190.6–209.3	1.66–1.77	12.71–13.95	[71]
T. saccharolyticum	Δ*nfnAB*	cellobiose	5	53.8	/	/	[114]
T. thermosaccharolyticum MJ1	Wild type	cellobiose/glucose/xylose	5	85.6/78.3/71.6	11.2/4.3/2.2	/	[58]
T. thermosaccharolyticum MJ1	Wild type	acid-pretreated sugarcane bagasse hydrolysate	~5	105.4–134.4	/	/	[58]
C. thermocellum JN4 and *T. thermosaccharolyticum* GD17	Wild type	microcrystalline cellulose	5	55.0	0.8	/	[128]
C. thermocellum and *T. aotearoense*	Wild type	pretreated sugarcane bagasse	40	50.1	/	/	[124]

Table 4. Butanol production by *Thermoanaerobacter* species.

Strain	Genotype	Substrate	Substrate Concentration (g/L)	Fermentation Mode	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Ref.
T. thermosaccharolyticum M5	Wild type	Xylan	20	CBP	1.17	/	/	[126]
T. thermosaccharolyticum M5, *C. acetobutylicum* NJ4	Wild type (co-culture)	Xylan	60	CBP	8.34	0.14	/	[126]
T. thermosaccharolyticum M5, *C. acetobutylicum* NJ4	Wild type (co-culture)	Xylan; untreated corncob	70; /	CBP	13.28; 7.61	/	0.079; 0.045	[129]
T. thermosaccharolyticum strain TG57	Wild type	Microcrystalline cellulose	28	Batch	1.93	0.20	0.005	[45]
T. thermosaccharolyticum strain TG57	Wild type	Beechwood xylan	30	Batch	3.63	0.23	0.019	[45]
T. thermosaccharolyticum strain TG57	Wild type	Xylose and cellobiose	/	Batch	6.21	0.28	/	[45]
T. thermosaccharolyticum strain TG57	Wild type	Glucose, xylose, and arabinose	/	Batch	7.33	/	/	[45]
T. saccharolyticum JW/SL-YS485	::*thl-hbd-crt-bcd-etfAB(Tt)-adhE2(Ca)*	Xylose	10	Batch	0.85	0.17	/	[127]
T. saccharolyticum JW/SL-YS485	*Δldh*::*thl-hbd-crt-bcd-etfAB(Tt)-adhE2(Ca)*	Xylose	10	Batch	1.05	0.21	/	[127]

4.3. Improvement of the Tolerance to Lignocellulose-Derived Inhibitors

T. saccharolyticum was able to utilize most of the carbohydrates in the hydrolysates of pretreated hardwood, indicating that the strain possessed all the proteins required for efficiently breaking down hemicellulose and could produce twice more ethanol from detoxified hydrolysate than that of untreated hydrolysate (31 g/L vs. 16 g/L) in fermenters [54]. It's reported that lignocellulose-derived inhibitors could trigger the accumulation of reactive oxygen species (ROS), resulting in cell damage. Some researches also suggested that inhibitors presented a redox challenge to cells [54,130]. Although the inhibitors such as HMF and furfural could be metabolized by strains like *T. saccharolyticum*, some inhibitors such as furfural could delay growth, and others such as vanillin could affect cell yield [54,76].To make full use of the lignocellulose, detoxification of lignocellulosic hydrolysates was usually necessary.

Activated carbon was used to reduce the toxicity of both the solid and the liquid feeds [49]. The treatment of the hydrolysate with diafiltration and/or 1% activated carbon resulted in much higher ethanol titers [54]. Tolerance of the strains to inhibitors was greatly improved by the presence of cysteine in the medium [54]. Additionally, acetic acid was also a kind of inhibitor. The ethanol production was stopped when acetic acid levels reached 10–14g/L [54]. Overliming was demonstrated as an effective method to detoxify polymeric hemicellulose [54]. An excess of calcium carbonate provided excellent buffering at a pH of 5.5, which is close to the optimum pH for *T. saccharolyticum* [49]. Since many inhibitors had greater negative effect in minimal media than in yeast extract containing media, addition of yeast extract seemed a simple way for alleviating the suppression [24,49].

Moreover, genetic modification also played a vital role in strengthening *Thermoanaerobacter* species against lignocellulose-derived inhibitors, as shown in Table 5. Elimination of the gene *perR* in *T. saccharolyticum* led to dramatical improvement in inhibitor tolerance and higher oxygen tolerance as well as higher ethanol titer [49,130]. The *pta/ack* genes seemed the key genes for the inhibitor tolerance phenotype and the dramatic inhibitor tolerance was conferred by restoring the *pta*/*ack* genes into a *pta*/*ack*(−) strain [49]. In strain SCUT27/Δ$argR_{1864}$, the improved concentration of intracellular ATP and NAD(H) provided more energy to respond the stresses, which gave the mutant the better cell viability to utilize lignocellulosic hydrolysates for enhancing ethanol production [44]. In addition, the transcriptome data between SCUT27/Δ$argR_{1864}$ and wild type showed that $ArgR_{1864}$ was the negative regulator for chaperonin synthesis. With $argR_{1864}$ knockout, the mutant showed the upregulated expression of DnaK-DnaJ-GrpE system and GroEL-GroES chaperonin, which not only endowed the strain with the enhanced ability to scavenge the reactive oxygen species (ROS), but also gave the mutant the capability to maintain better cell growth, xylose consumption, acetic acid and ethanol production under the stress of various lignocellulose-derived inhibitors [131].

It was reported that *T. saccharolyticum* was inhibited by monosaccharides at concentrations higher than 40 g/L, and with methylglyoxal synthase deletion, the mutant could grow at 100 g/L glucose and produce more ethanol from hardwood hydrolysate [49]. Furthermore, during continuous cultures of *T. thermosaccharolyticum*, the cessation of growth and fermentation at feed exceeding 70 g/L xylose seemed to be attributed to salt formed by neutralizing organic acid rather than the toxicity of ethanol [132]. Notably, salt inhibition could be avoided by introduction of the urease operon [67].

Table 5. Gene sites engineered for enhanced tolerance against inhibitors in *Thermoanaerobacter* species.

Gene	Function	Engineering Strategy	Phenotype of Mutats	Ref.
perR	repressor of oxidative stress response	Knockout	upregulated oxidative stress response, improved inhibitor and oxygen tolerance	[130]
pta/ack	synthetic pathway of acetic acid	Complement in mutant of *pta/ack*	Improved inhibitor tolerance	[49]
argR	Arginine repressors and as a global regulator	Knockout	enhanced stress tolerance and ethanol formation; upregulated expression of DnaK-DnaJ-GrpE system and GroEL-GroES chaperonin	[44]
ureABCDEFG	Urease operon	Introduction	Avoid of salt inhibition	[67]
rex	redox-sensing transcriptional repressor	Knockout	improvement of total ATP concentration and the cofactors NADH and NAD+ concentrations	[90]
mgs	Methylglyoxal synthase	Knockout	Reduce the glucose toxicity	[49]

5. Applications of *Thermoanaerobacter* Species for Various Bioprocessing

When enzymatic hydrolysis is adopted for cellulosic ethanol production, different levels of process integration can be visualized: (i) separate (or sequential) hydrolysis and fermentation (SHF), where the enzymes (cellulases) are used separately from fermentation tanks; (ii) simultaneous saccharification and fermentation (SSF), which consolidates enzymatic hydrolysis with the fermentation of hexose and pentose sequentially; (iii) simultaneous saccharification and cofermentation (SSCF), which further combines the fermentation of hexose and pentose together; and (iv) consolidated bioprocessing (CBP), where cellulases and ethanol are produced in a single reactor [33]. For *Thermoanaerobacter* species, cellulose hydrolysis is the main limiting factor in the progress of fermentation [34].

A fed-batch fermentation with *T. saccharolyticum* M2886 was performed and 50 g/L ethanol after 60 h cultivation under pretreated hardwood was obtained in SHF mode [49]. 37 g/L ethanol was produced by strain ALK2 in SHF at 50°C which had a 2.5-fold decrease in cellulase loading compared with that of *S. cerevisiae* at 37 °C [34]. For SSF, mesophiles ferment well at ≤37 °C and thus require substantially higher cellulase loadings compared with the thermophiles cultured under 50~60 °C (the optimal temperature for both enzymes activity and the growth of *Thermoanaerobacter*) [34,54]. Pre-hydrolysis for a long time facilitates the cellulose conversion, but the high initial sugar concentrations >50 g/L (usually due to high initial enzyme dose) would inhibit the cells growth [54]. SSF of avicel with *T. saccharolyticum* in batch mode at an initial concentration of 50 g/L after pretreatment with a commercial cellulase showed the better performance than that that of yeast (2.5-fold increase of cellulase loading). And further reductions in cellulase loading could be anticipated as more thermostable cellulases are developed [34,133]. Under the optimal substrate "mock hydrolysate" and conditions such as temperature, pH-control strategy, inoculum size, cellulase mixture and feeding regimen, *T. saccharolyticum* could produce over 61 g/L ethanol in thermophilic SSF [54]. Moreover, 31 g/L ethanol was produced in SSF of pre-treated hardwood and 26 g/L ethanol was obtained with a hardwood hemicellulose extract [49]. Fermentation conditions were also developed to get the highest possible ethanol titer in a SSCF configuration under the conditions expected from pre-treated hardwood (100 g/L purified cellulose, 10 g/L acetic acid, 35 g/L xylose and 20 g/L glucose), and more than 60 g/L ethanol (greater than 90% yield) was achieved at 93 h cultivation by strain M1442 [49]. Additionally, cell lysis behavior was observed when the strain grew too fast, but it could be prevented under mixed sugars with a slower growth rate, or in the medium with high concentration of magnesium or low pH [54].

A preferred CBP system can directly convert both cellulose and hemicellulose into target products without the costly hydrolytic enzyme cocktail. Cellulolytic strains like *C. thermocellum* are necessary to be used to hydrolyze and ferment all of the sugars in the broth with *Thermoanaerobacter* species. It was reported that the coculture of *C. thermocellum* JN4 and *T. thermosaccharolyticum* GD17 could significantly improve the efficiency of cellulosic biofuel [134]. Biodegradation of sugarcane bagasse (SCB) was remarkably improved with the addition of non-ionic surfactant (etc. Triton X-100) to the CBP system with *C. thermocellum* [135]. The applications of CBP for hydrogen production have been shown above (section 4.2.2). Lynd et al. also listed some detailed advances in CBP using *C. thermocellum* and *T. saccharolyticum* [136]. While these production schemes represent increasing levels of simplification through process consolidation, consolidation of multiple steps often results in a loss of process efficiency. Improving the efficiency of individual step, such as cellulose hydrolysis and ethanol fermentation, is still the important task for the development of economically feasible cellulosic biofuel.

6. Conclusions

In this paper, the traits of *Thermoanaerobacter* species on substrate utilization, their current platforms of genetic operation, and the strategies for enhanced biofuels (ethanol, hydrogen and butanol) production were reviewed. Numerous ways have been developed to enhance the sugar consumption and biofuel production in *Thermoanaerobacter* species. However, the production of ethanol is far less than that of *S. cerevisiae* and *Z. mobilis.* Achievement of biobutanol with high titer is still difficult due to the overwhelming metabolic stress. Further research should be performed as follows to establish efficient microbial cell factories for the genus *Thermoanaerobacter* to produce biofuels or other bioproducts:

(i) Better understand the genes and pathways involved in carbohydrate transport and metabolism, and address the mechanism of the co-utilization between glucose and xylose, which can shorten the fermentation period and make full use of the lignocellulosic materials.

(ii) Further develop effective elements of genetic operation such as useable fluorescent proteins for *Thermoanaerobacter* species, and technologies in large-scale engineering like CRISPR*i* and CRISPR*a*, for conveniently analyzing metabolic flux, engineering the high-producing strains and exploring the physiological mechanism of the bacteria.

(iii) The whole-cell models of *Thermoanaerobacter* species should be built to analyze the cellular networks and find key bottlenecks in biofuels production. Then the corresponding metabolic engineering strategies could be used for enhancing titer and yield of the products.

(iv) Tolerance to the inhibitors, high concentration of substrates and products, always poses a great challenge for *Thermoanaerobacter* species. It is necessary to reveal the mechanism and lay the fundation to improve the resistance of cells to several inhibitors for higher biofuels production.

(v) Optimize the fermentation process and construct microbial consortia to reduce the metabolic burden. More strategies and techniques should be practiced on perspective applications of *Thermoanaerobacter* species for consolidated bioprocessing.

(vi) Other valuable biochemicals and bioproducts such as poly-3-hydroxybutyrate (PHB), polylactic acid (PLA) and fatty acid esters could be further developed in *Thermoanaerobacter* species once getting the responding thermostable enzymes.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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