




Microbial application of thermophilic *Thermoanaerobacterium* species in lignocellulosic biorefinery

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Abstract

Recently, thermophilic *Thermoanaerobacterium* species have attracted increasing attentions in consolidated bioprocessing (CBP), which can directly utilize lignocellulosic materials for biofuels production. Compared to the mesophilic process, thermophilic process shows greater prospects in CBP due to its relatively highly efficiency of lignocellulose degradation. In addition, thermophilic conditions can avoid microbial contamination, reduce the cooling costs, and further facilitate the downstream product recovery. However, only few reviews specifically focused on the microbial applications of thermophilic *Thermoanaerobacterium* species in lignocellulosic biorefinery. Accordingly, this review will comprehensively summarize the recent advances of *Thermoanaerobacterium* species in lignocellulosic biorefinery, including their secreted xylanases and bioenergy production. Furthermore, the co-culture can significantly reduce the metabolic burden and achieve the more complex work, which will be discussed as the further perspectives.

Key points

- *Thermoanaerobacterium* species, promising chassis for lignocellulosic biorefinery.
- Potential capability of hemicellulose degradation for *Thermoanaerobacterium* species.
- Efficient bioenergy production by *Thermoanaerobacterium* species through metabolic engineering.

Keywords *Thermoanaerobacterium* · Thermophile · Consolidated bioprocessing · Lignocellulose · Biorefinery

Introduction

Lignocellulosic biorefinery is a sustainable multi-step process, which can achieve the conversion of lignocellulosic materials into bioenergy and biochemicals (Calvo et al. 2018). As one of the most abundant and renewable resources in the world,

lignocellulose can be widely found in agricultural and industrial waste residues, such as bagasse, corn stalks, wheat stalks, and wood chips. Besides, as a ubiquitous energy source, lignocellulose not only provides energy security but also minimizes greenhouse gas emissions, which makes it an attractive material in biorefinery (Chatalova and Balmann 2017; Yadav et al. 2019). The major compositions of lignocellulose are cellulose, hemicellulose, and lignin. Among them, cellulose is a glucose polymer linked by β -(1 → 4)-glycosidic bonds, which usually accounts for 40–50% of the total lignocellulose. Hemicellulose accounts for 15–35% of plant biomass, which is a heterogeneous polymer composed of five different types of pentose or hexose, including D-xylose, L-arabinose, D-galactose, D-mannose, and D-glucose. Lignin is a complex natural polymer, which is widely present in plant cells (Menon and Rao 2012; Ravindran and Jaiswal 2016). However, due to the cross-linked structure, lignocellulosic materials are difficult to be hydrolyzed by hydrolases (Mirmohamadsadeghi et al. 2021; Ravindran and Jaiswal 2016). Pretreatment before enzymatic hydrolysis is a key step to improve the

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biodegradability of lignocellulose, including physical pretreatment (grinding, etc.), chemical pretreatment (acid, alkali, oxidant), enzymatic hydrolysis, and the combination of these pretreated methods (ammonia fiber explosion etc.) (Frigon et al. 2012; Zheng et al. 2017). However, the high cost hinders their large-scale applications (Sołowski et al. 2020).

Consolidated bioprocessing (CBP) is considered as a promising method for biofuels production from lignocellulose, which combines hydrolytic enzymes production, lignocellulose hydrolysis, and microbial fermentation in one tank (Jiang et al. 2020a). The combination of these steps into one process configuration is encouraging; however, adoption of appropriate chassis microorganisms is still the prime requirement. Thermophilic anaerobes refer to those whose optimal growth temperature is higher than 50 °C, which will be beneficial to increase reaction kinetics and lignocellulose degradation efficiency (Fu et al. 2015; Singh et al. 2020). Compared with mesophilic microorganisms, higher cultural temperature can also reduce the risk of microbial contamination (Jiang et al. 2017). Additionally, the thermophilic process can reduce cooling costs and further promote downstream product recovery (Bhalla et al. 2013; Tukacs-Hajos et al. 2014).

Thermoanaerobacterium species are a group of endospores that form swimming, rod-shaped, and obligate anaerobic prokaryotes (Jiang et al. 2018). This genus can directly utilize hemicellulose, some of which can even degrade cellulose (Jiang et al. 2019; O-Thong et al. 2008). Thus, *Thermoanaerobacterium* species show promise as potential chassis microorganisms for achievement of lignocellulosic biorefinery. Accordingly, this review will comprehensively investigate the hydrolases and potential functions of *Thermoanaerobacterium* species as industrial workhorses for lignocellulosic material degradation and chemicals production, and the analysis of key enzymes and metabolic pathways will provide guidance for subsequent engineering and applications. Additionally, the perspective applications via CBP by using microbial consortia will also be discussed, which is beneficial to further improve the efficiency of lignocellulosic biorefinery.

Hydrolases of hemicellulose degradation secreted by thermophilic *Thermoanaerobacterium* species

The efficient hydrolysis of lignocellulosic biomass into fermentable sugars is the primary step for biorefinery. However, due to the complex structure of lignocellulose, a large number of hydrolytic enzymes are required for the effective saccharification process. Glycoside hydrolases play the synergistical roles in lignocellulose degradation, including cellulase, hemicellulase, and pectinase, which can be produced by various bacteria and fungi to hydrolyze the

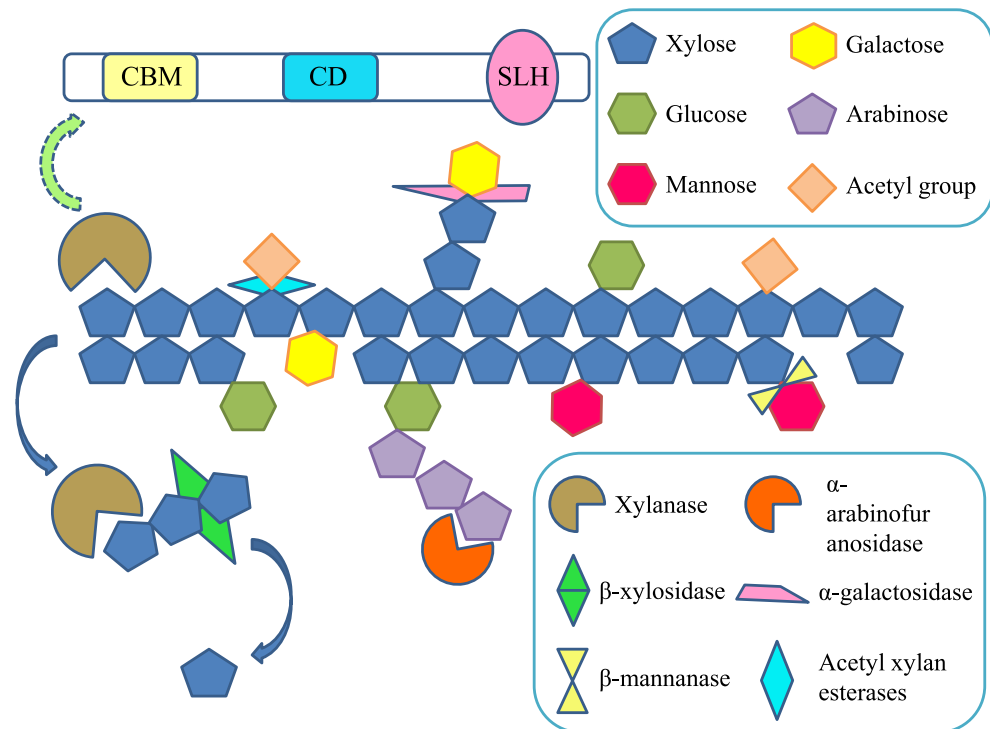
glycosidic bonds between carbohydrates or carbohydrates and non-carbohydrates (Singh et al. 2020).

As known, *Thermoanaerobacterium* species can quickly decompose lignocellulosic materials especially hemicellulose. Xylan is the main constituent of hemicellulose, and xylanase and β -xylosidase are the two crucial xylanolytic enzymes involved in the biodegradation of xylan. Xylanase can randomly cleave the β -1,4 bonds in the backbone, transforming xylan into xylo-oligosaccharides with different lengths. The mixture of xylo-oligosaccharides is then hydrolyzed by β -xylosidase into xylose molecules (Chadha et al. 2019; Lee et al. 1993b). Generally, xylanases are divided into several glycoside hydrolase (GH) families (5, 7, 8, 10, 11, 26, 30, and 43), which can be produced by a variety of microorganisms such as extremophiles, bacteria, and yeast (Chadha et al. 2019). In terms of *Thermoanaerobacterium* species, the major secreted xylanases belong to GH10 and contain carbohydrate-binding module (CBM) and S-layer homology (SLH) domain (Fig. 1).

CBM is defined as a continuous amino acid sequence with discreet folds and carbohydrate binding activity, which was once classified as cellulose binding domain (CBD). Based on bioinformatics and molecular biology technology, a new viewpoint called the “modular structure” of xylanase is proposed. This view points out that these xylanases include at least one catalytic module and/or CBM. Different CBMs act as targeting agents to attach the catalytic module of enzymes to the corresponding polysaccharide component of lignocellulosic biomass. The complexity of enzymes depends on the number of catalytic modules and CBMs on each polypeptide chain (Singh et al. 2020). Furthermore, the xylanases secreted by *Thermoanaerobacterium* species also have the S-layer homology (SLH) domain, which is a C-terminal triplet sequence related to bacterial cell surface proteins. Sequence analysis indicated that the SLH domain can fix the protein on the cell surface to promote the binding with enzyme complex (Lemaire and Ohayon 1995).

A newly obtained *Thermoanaerobacterium* sp. M5 is a thermophilic and butanogenic bacterium with the ability of xylan degradation and butanol production. The final metabolic products of strain M5 contain butyrate, acetate, ethanol, butanol, and H₂. According to the phylogenetic analysis, *Thermoanaerobacterium* sp. M5 shows 99% similarity with *T. thermosaccharolyticum* KKKU19 (Jiang et al. 2018). The xylanase (D9TMZ9) that was annotated in strain M5 contains a GH10 domain, three CBMs, and three SLH domains. The GH10 domain is responsible for the random hydrolysis of β -(1,4)-xylosidic bonds within the insoluble xylan backbone. And the two domains of CBMs and SLHs facilitate the binding between the enzyme and xylan, contributing to the efficient xylan degradation (Jiang et al. 2018). *T. thermosaccharolyticum* DSM 571 is a rigorous anaerobe that can grow on various hexose and pentose sugars at temperatures between 37 and 75 °C (Li et al. 2014), and the

Fig. 1 Enzyme system involved in the degradation of hemicellulose and the schematic representation of the “modular structure” of xylanase in *Thermoanaerobacterium* species. CBM, carbohydrate binding module; CD, catalytic domain; SLH, S-layer homology domain



detected major metabolic products are H_2 and biofuels. XYN is a heat-resistant, cellulase-free xylanase secreted by the strain DSM 571, which is an endoxylanase with the capability of xylan degradation instead of xylobiose. The catalytic domain of XYN belongs to the GH10 family, and three CBMs strengthen the connection between the enzyme and insoluble substrates. Additionally, the anchoring of the enzyme on bacterial surface layer is mediated by the three SLH domains. It has been found that 65 °C and pH 6.5 are the most suitable conditions for the enzyme activity of XYN and the half-life is 1 h at 71 °C, indicating the good thermal stability of XYN (Li et al. 2014).

T. saccharolyticum is another extensively studied species for xylan degradation and form ethanol and/or lactic acid as major end products, as well as lower levels of H_2 - CO_2 and acetate (Lee et al. 1993a). It is a potential chassis microorganism for ethanol production. For example, *T. saccharolyticum* JW/SL-YS485 is a strain that came from a site in Yellowstone National Park, which can grow at a wide range of pH from 3.85 to 6.35 and a temperature within the scope of 30 to 66 °C (Shao et al. 1995). TsXynA is a cell-associated endoxylanase extracted from *T. saccharolyticum* JW/SL-YS485, which belongs to the GH10 family (Han et al. 2013). The enzyme has a large molecular weight, which is composed of two subunits (24 and 180 kDa). The 180-kDa subunit was deemed as a glycoprotein containing 6% of carbohydrates. The 24-kDa one is a breakdown product of the 180-kDa protein which has an N-terminal sequence and was considered to be

necessary for maximum enzymatic activity (Liu et al. 1996). A catalytic domain, two CBMs, and three S-layer repeats at the C-terminal constitute the TsXynA. This enzyme also exhibited excellent thermotolerance, which the optimal temperature was 80 °C, and the half-life at 77 °C was 1 h (Han et al. 2013). Similar with TsXynA, XynFCB, an endo-1,4- β -xylanase generated by strain *T. saccharolyticum* NTOU1 also belongs to the GH10 family. Especially, the thermophilic marine bacterium *T. saccharolyticum* NTOU1 was acquired from an oceanic hydrothermal vent, resulting in XynFCB was also proven to be a salt-tolerant enzyme (Hung et al. 2011). In details, the activity of XynFCB was shown an increase in 0–12.5% (w/v) NaCl, and maintained 67% of activity in 15% (w/v) NaCl after a 48-h incubation. Resemble with the overall growth conditions of strain NTOU1 (45–78 °C and pH 5.0–9.0), XynFCB operated well between 55 and 65 °C and pH within the extent of 6.0 to 6.5, and its half-life was 55 min at 65 °C (Hung et al. 2011).

In addition to *T. thermosaccharolyticum* and *T. saccharolyticum* that are commonly reported anaerobic species with the xylanases secretion, *T. aotearoense* is another studied one for xylan degradation and their xylanases also have such a modular structure. *T. aotearoense* SCUT27 is a Gram-positive, strictly anaerobic, thermophilic bacterium that was recently isolated from a hot spring in China and developed as a biocatalyst for the production of ethanol, H_2 , and lactic acid (Ai et al. 2014). It possesses the capability of utilization xylan as the sole carbon source by secretion of a

Table 1 Comparison of properties of different thermophilic *Thermoanaerobacterium* species

Strains	Growth temperature	Growth pH	Major metabolic products	Xylanases			References	
				Names	Modular structure	Optimum temperature		Optimum pH
<i>Thermoanaerobacterium</i> sp. M5	/	/	Butyrate Acetate Ethanol Butanol H ₂ H ₂ Biofuels	D9TMZ9	1 GH10 domain 3 CBMs 3 SLH domains	/	/	(Jiang et al. 2018)
<i>T. thermosaccharolyticum</i> DSM 571	37–75 °C	/		XYN	1 GH10 domain 3 CBMs 3 SLH domains	65 °C	6.5	1 h at 71 °C (Li et al. 2014)
<i>T. saccharolyticum</i> JW/SL-YS485	30–66 °C	3.85–6.35	Ethanol and/or Lactic acid, H ₂ -CO ₂ and acetate	TsXynA	1 GH10 domain 2 CBMs 3 SLH domains	80 °C	6.3	1 h at 77 °C (Han et al. 2013)
<i>T. saccharolyticum</i> NTOU1	45–78 °C	5.0–9.0		XynFCB	1 GH10 domain 3 SLH domains	63 °C	6.4	55 min at 65 °C (Hung et al. 2011)
<i>T. aotearoense</i> SCUT27	/	/	Ethanol H ₂ Lactic acid	XynA	1 GH10 domain 4 CBMs 3 SLH domains	/	/	(Huang et al. 2015)

Table 2 H₂ production by thermophilic *Thermoanaerobacterium* species

Strains	Substrates	Hydrogen production (mM)	Hydrogen yield (mL H ₂ /g xylan)	Hydrogen production rate (mL H ₂ /L•h)	Xylanase activity (units/mL)	References
<i>T. thermosaccharolyticum</i> KKU-ED1	Xylan	/	120.05	11.53	0.41	(Sariipan and Reungsang 2013b)
<i>T. thermosaccharolyticum</i> KKU-ED1	Sugarcane bagasse (SCB)	/	5.77	0.66	0.26	(Sariipan and Reungsang 2013b)
<i>Thermoanaerobacterium</i> sp. strain F6	Xylan	370.7	/	/	/	(Jiang et al. 2019)
<i>Thermoanaerobacterium</i> sp. strain F6	Corn cob	66.7	/	/	/	(Jiang et al. 2019)

xylanase: XynA, which was attested containing one GH10 domain, four CBMs, and three SLH domains (Huang et al. 2015; Yang et al. 2013).

Taken together, the xylanases produced by the *Thermoanaerobacterium* species have a similar “modular structure” and is generally with good heat resistance (Table 1), which renders them to effectively hydrolyze xylan in lignocellulose and shows great potential for applications in lignocellulosic biorefinery.

Application of *Thermoanaerobacterium* species in bioenergy production

As discussed above, the bioenergy production from the lignocellulosic biomass is a potential way to reduce the demand for carbon-intensive fossil fuels (Herring et al. 2016). Currently, microbial catalysts that can directly convert cellulose and xylan into biofuels are being developed. Owing to the capability of hydrolyzing and fermenting soluble cellodextrins and xylan, *Thermoanaerobacterium* species is a promising chassis microorganism in cellulosic biofuel industry (Shaw et al. 2011). Unlike *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and thermophilic *Clostridium thermocellum*, which have only been used hexose sugars for bioenergy production, but not the pentose sugars, *Thermoanaerobacterium* species are hemicellulolytic thermophilic anaerobes that can utilize pentose sugars derived from hemicellulose for hydrogen and ethanol production. Thus, the relatively high growth and metabolic rates of *Thermoanaerobacterium* species growing on both cellulose and hemicellulose indicate they are the potential microorganisms for lignocellulosic biorefinery (Barnard et al. 2010).

Biohydrogen production from lignocellulose by *Thermoanaerobacterium* species

On account of the high energy content (142 kJ/g) and the environmentally friendly feature as its combustion results only in water vapors, hydrogen is being received an attention as the fuel of future. Due to the high conversion efficiency, recyclability and nonpolluting nature, sustainable biological hydrogen has enormous potential as an alternative energy, and the development of its production process is of great significance (Sinha and Pandey 2011). Lignocellulosic biohydrogen is a promising renewable energy that might be an ideal substitute for unsustainable fossil fuel-based energy and can be produced by anaerobic bacteria degrading carbohydrate-rich substrates (O-Thong et al. 2008; Wang et al. 2016). Among these H₂ producers, mesophilic bacteria are the most extensively studies ones. However, it is reported that the yields of H₂ were enhanced when using thermophiles or extreme thermophiles in the fermentation process (Kadar et al. 2004; van Niel et al. 2002). The reason why is that a higher temperature can provide favorable thermodynamics conditions which will not only speed up the production rate of H₂, but also minify the variety of end products in fermentation (Schonheit and Schafer 1995). Therefore, compared with mesophilic bacteria, thermophilic bacteria are considered to have a greater development prospect in the production of H₂ (O-Thong et al. 2008). In recent years, there are many researches of using the thermophilic *Thermoanaerobacterium* species in H₂ manufacturing that attract widespread attention ascribed to its direct exploit of lignocellulose.

T. saccharolyticum KKU-ED1 is a thermophilic hydrogen producing strain which was isolated from elephant dung (Sariipan and Reungsang 2013b). It has a broad substrates spectrum for hydrogen production including glucose, xylose, fructose, starch, xylan, and mixed xylose/arabinose (Sariipan

and Reungsang 2013a). As described above, xylanase is the key enzyme in the depolymerization of xylan into xylose, and the highly activity of xylanase by *T. saccharolyticum* KKU-ED1 contributes to the hemicellulosic hydrogen production. After the optimization of the cultural conditions, the initial pH of 7.0, temperature of 55 °C, and xylan concentration of 15 g/L were the best conditions for strain KKU-ED1 to produce hydrogen from xylan. Finally, the hydrogen yield, productivity, and xylanase activity were achieved at 120.05 mL H₂/g xylan, 11.53 mL H₂/L·h, and 0.41 U/mL, respectively (Table 2). Considering as the excellent utilization capability of xylan in *T. saccharolyticum* KKU-ED1, the sugarcane bagasse (SCB) riched hemicellulose was attempted as the carbon source for hydrogen fermentation. While due to the more complex structure of the SCB, the hydrogen production has slightly declined to some extent (Saripan and Reungsang 2013b). Compared to 0.41 U/mL, the xylanase activity decreased to 0.26 U/mL, and the hydrogen yield and productivity were decreased at 5.77 mL H₂/g xylan (1.39 mL H₂/g SCB) and 0.66 mL H₂/L·h, respectively (Table 2).

A newly isolated thermophilic bacterium *Thermoanaerobacterium* sp. strain F6 showed relatively high hydrogen generation capability from wide substrate spectrums, including hemicellulosic materials and cellulosic materials (Jiang et al. 2019). Compared with cellulosic feedstock, strain F6 produced higher hydrogen production from hemicellulosic substrates, such as xylan. When the concentration of xylan increased to 60 g/L, the maximum cumulative hydrogen production increased at 370.7 mM (Table 2). Considering as the better hemicellulose degradation capability, corn cob, a kind of agricultural residues whose contents of hemicellulose can reach 38% was investigated as the carbon source to produce hydrogen (Mullen et al. 2010; Shen et al. 2015). From 30 to 60 g/L of corn cob, the accumulated hydrogen was 41.7 and 66.7 mM, respectively (Table 2). Despite the final hydrogen production from lignocellulosic biomass is much less than xylan, these researches demonstrated the feasibility of hydrogen production via CBP by using lignocellulosic materials without any hydrolysis pretreatment (Jiang et al. 2019).

Bioethanol production from lignocellulose by *Thermoanaerobacterium* species

Ethanol is the first commercialized cellulosic biofuel, which produces water and carbon dioxide when fully burned (Herring et al. 2016). Using the thermophilic bacteria to produce ethanol though biological fermentation shows several advantages: (i) elevated temperatures can prevent the contamination of mesophilic bacteria; (ii) ethanol may self-distill to avoid the generally low tolerance of these bacteria to ethanol and reduce the cost of separation; (iii) the high cultivation temperature can also reduce the pressure of the cooling system

and reduce the cooling cost (Almarsdottir et al. 2012). *Thermoanaerobacterium* species with the capability of hemicellulose degradation are the promising candidate for ethanol production from lignocellulosic biomass. However, the natural *Thermoanaerobacterium* species generally produce low level of ethanol. Therefore, it is very necessary to improve the final ethanol titer through genetic engineering, and classical mutagenesis/selection et al.

T. saccharolyticum possesses natural susceptibility and recombination ability, making gene manipulation relatively easy (Herring et al. 2016). These engineering attempts in *T. saccharolyticum* can be divided into two categories: the first mainly focuses on the elimination of natural competitive metabolic pathways (such as organic acid production), usually combined with adaptive evolution to accelerate the growth of engineered strains (Shaw et al. 2008b). The second type of engineering attempts focus on the introduction of heterologous genes into *T. saccharolyticum* (Shaw et al. 2012).

T. saccharolyticum has the capability to degrade lignocellulose and produce bioethanol. However, accompanied with the production of ethanol, some organic acids have also been produced which lessen the yield of the primary product (Shaw et al. 2008a). Analysis of the central metabolic pathways in *T. saccharolyticum* (Fig. 2), it can be noticed that ethanol is converted from pyruvate. From pyruvate to ethanol, there are two main branched fermentation pathways leading to lactic acid and acetic acid, separately, which are the major hurdles for the efficient ethanol production. In terms of lactic acid, it is formed from pyruvate by lactate dehydrogenase, while with regard to acetic acid, its synthesis from pyruvate is through a pathway containing pyruvate:ferredoxin oxidoreductase (POR), phosphate acetyltransferase, and acetate kinase (Shaw et al. 2008b). Based on the understanding of these pathways, the researchers have come up with gene knockout. By knocking out genes involved in the formation of lactic acid and acetic acid, the two branched fermentation pathways can be blocked. And at the same time, new metabolic flux distribution might be constructed, which may lead more carbon flux available for ethanol synthesis. Three genes (*L-ldh*, *pta*, *ack*) related to the by-products production were knockout, and three mutants of *T. saccharolyticum* were obtained, including $\Delta L-ldh$, $\Delta ack \Delta pta$, and $\Delta ack \Delta pta \Delta L-ldh$ one which was designated as strain ALK1. Analysis the fermentation products of these strains in xylose-grown cultures, the $\Delta L-ldh$ mutant did not produce detectable lactic acid, and the $\Delta ack \Delta pta$ mutant did not produce acetic acid, while both of them have an increased yield of ethanol from pyruvate. As to strain ALK1, the results showed that it produced ethanol as the only detectable organic product. After continuously cultivating strain ALK1 for near 3000 h with the progressively enhanced fed xylose concentrations, a strain that have better xylose consumption ability in both batch and continuous culture have been acquired and was named ALK2. The strain ALK2 was

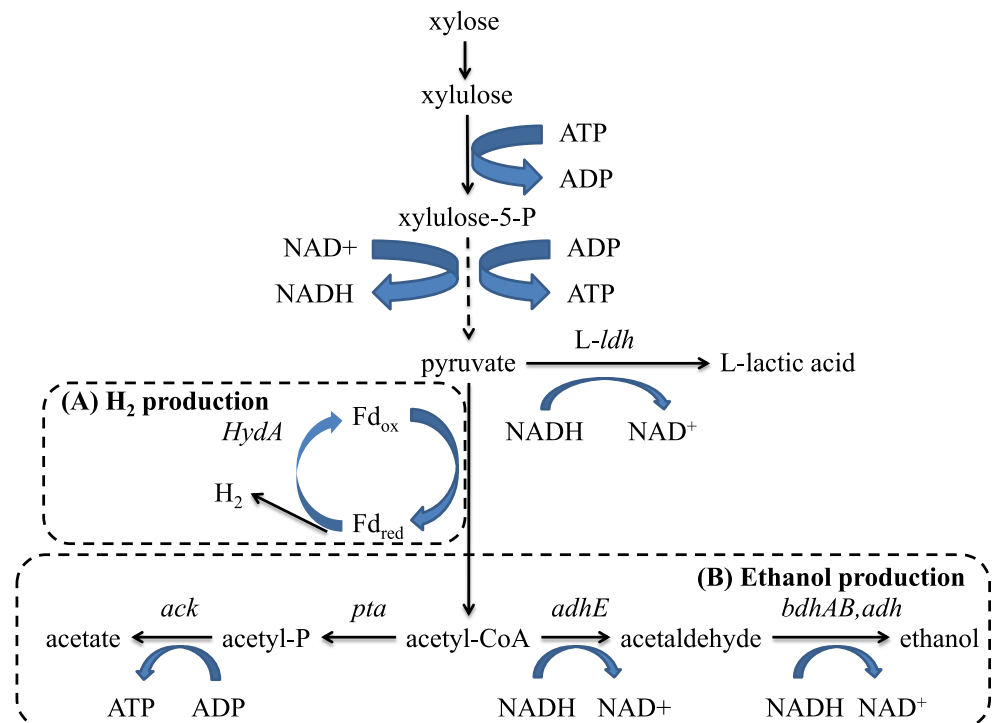
Table 3 Ethanol production by thermophilic *Thermoanaerobacterium* species

Strains	Fermentation mode	Substrates	Nitrogen sources	Concentration (g/L)	Yield (g/g)	References
<i>T. saccharolyticum</i> ALK2	Continuous	Xylose	/	33	/	(Shaw et al. 2008b)
<i>T. saccharolyticum</i> ALK2	Fed-batch	Mixed sugars (50 g/L)	/	37	/	(Shaw et al. 2008b)
<i>T. saccharolyticum</i> M1051	/	Lignocellulose	Urea	54.3	/	(Shaw et al. 2012)
<i>T. saccharolyticum</i> M1051	/	Lignocellulose	Ammonium sulfate	25.9	/	(Shaw et al. 2012)
<i>T. aotearoense</i> SCUT27	/	Rice straw	/	0.65	0.07	(Qu et al. 2020)
<i>T. aotearoense</i> SCUT27/ Δ argR1864	/	Rice straw	/	5.59	0.35	(Qu et al. 2020)
<i>T. aotearoense</i> SCUT27	/	Corn cob	/	0.71	0.08	(Qu et al. 2020)
<i>T. aotearoense</i> SCUT27/ Δ argR1864	/	Corn cob	/	5.40	0.37	(Qu et al. 2020)
<i>T. aotearoense</i> SCUT27	/	Sugarcane Bagasse	/	0.66	0.09	(Qu et al. 2020)
<i>T. aotearoense</i> SCUT27/ Δ argR1864	/	Sugarcane Bagasse	/	5.24	0.40	(Qu et al. 2020)

used for ethanol fermentation in both of continuous culture and fed-batch culture. In terms of continuous culture, the mean ethanol yield of strain ALK2 at pH 5.2–5.4 was 0.46 g ethanol/g xylose, and the maximum ethanol concentration and volumetric productivity was 33 g/L and 2.2 g/L·h, respectively (Table 3). Compared with some other xylose-utilizing recombinant organisms such as *S. cerevisiae* and *Z. mobilis*, whose ethanol concentration that finally harvested was under

25 g/L (Kuyper et al. 2005; Lawford and Rousseau 1999; Sedlak and Ho 2004), *T. saccharolyticum* ALK2 exhibited favorably ethanol productivity. When it comes to fed-batch culture, strain ALK2 was cultivated in a 1-L fed-batch fermenter from 50 g/L of mixed sugars including glucose, xylose, galactose, and mannose. Differs from some mesophilic strains which use glucose preferentially to xylose, strain ALK2 can utilize glucose and xylose simultaneously and co-ferments

Fig. 2 The metabolic pathway for H₂ and ethanol synthesis in *Thermoanaerobacterium* species. **A** The H₂ formation pathway in *Thermoanaerobacterium* species. **B** The ethanol formation pathway in *T. saccharolyticum*. L-ldh, L-lactate dehydrogenase; Fd_{ox}, pyruvate:ferredoxin oxidoreductase oxidized; Fd_{red}, pyruvate:ferredoxin oxidoreductase reduced; HydA, hydrogenase; pta, phosphotrans acetylase; ack, acetate kinase; adhE, acetaldehyde dehydrogenase; bdh, butanol dehydrogenase; adh, alcohol dehydrogenase



mannose and galactose. The final ethanol concentration obtained from the mixed sugars reached 37 g/L, and the maximum ethanol production rate was 2.7 g/L·h, with the average of 1.5 g/L·h (Table 3) (Shaw et al. 2008b).

Because lignocellulosic raw materials are relatively lack of nitrogen to support the growth of fermenting organisms, low-cost, easy-to-use nitrogen sources are particularly important for lignocellulose conversion. In industrial fermentation, nitrogen including ammonia, ammonium salts, and amino acids can be supplied to support the growth and metabolism of microorganisms. The nitrogen cost of urea is lower than other nitrogen sources, while organisms using urea must have urease (EC 3.5.1.3) or urea amidolyase to catalyze urea into carbon dioxide and ammonia. The urease gene *ureABCDEFGF* from *C. thermocellum* ATCC 27405 (Cthe_1812-Cthe_1818) can be heterologously expressed in *T. saccharolyticum* strain. After amplified by PCR, this 5.1-kb fragment was cloned into a chromosomally integrating expression vector in *T. saccharolyticum*. Finally, one strain which was re-isolated after 2–3 transfers in TSD1 minimal medium had been verified for the existence of the urease genes through PCR, and was named M1051. The strain M1051 was cultured in a complex medium containing 8.5 g/L yeast extract and produced 15 g/L of ethanol. After addition of urea or ammonium sulfate to the complex medium, the ethanol production of strain M1051 was increased, indicating that the yeast extract itself is not sufficient as a nitrogen source for cells. When using 90 mM urea (5.4 g/L) as the main nitrogen source, the cultured strain produced 54.3 g/L of ethanol, and the fermentation ended at pH 6.7. In contrast, when 90 mM ammonium sulfate was used as the nitrogen source, 25.9 g/L of ethanol was obtained, and the fermentation pH ended at 4.0 (Table 3). In response to the difference in the obtained ethanol titers, the researchers pointed out that this may have to do with the movement of pH in the medium. It is believed that the non-metabolized ammonium ions which are hydrolyze from urea could elevate the medium pH, combined with the ability of urea to provide metabolizable nitrogen without decreasing the medium pH that leads to higher ethanol titers. However, as for ammonium sulfate, its metabolism drops the pH of the medium to a level which is not conducive to the continued ethanol fermentation by *T. saccharolyticum*. In conclusion, the introduction of heterologous urease genes into the *T. saccharolyticum* strain not only allows it to utilize low-cost nitrogen sources, but also achieve higher ethanol titers (Shaw et al. 2012).

T. aotearoense SCUT27 exhibits the capability of utilization a wide spectrum of carbohydrates including glucose, xylose, and xylan and can produces biofuels such as hydrogen and ethanol. This ability renders the strain an attractive bacterium for the conversion of lignocellulosic biomass to ethanol (Cai et al. 2011). Although SCUT27 can co-utilize some sugars derived from lignocellulosic biomass, the existence of

carbon catabolite repression (CCR) cannot be neglected. For example, it was reported that to some extent, the utilization of xylose by SCUT27 was repressed by the presence of glucose (Zhu et al. 2015). Since xylose approximately accounts for 30% of the total carbohydrates of most lignocellulose biomass, it is crucially important to heighten the ability of the use of xylose in lignocellulosic biorefinery (Qiu et al. 2018). ATP is essential in both the uptake and the catabolism of xylose, so higher energy level in the cell can be conducive to the utilization of xylose (Qu et al. 2020). Besides, the redox balance also plays a considerable role in the substrate utilization (Salusjarvi et al. 2013). Arginine is regarded as a precursor of energy since it can provide ATP through the arginine deiminase (ADI) pathway (De Angelis et al. 2002). Arginine repressor (ArgR), the homohexamer which activates various arginine catabolic pathways and suppress the biosynthesis of arginine, had been originally reported to involved in several key metabolic pathways such as amino acid metabolism, carbohydrate, nucleotide, and energy metabolisms (Cho et al. 2012), all of which have vital impacts on the redox balance. Owing to the multifunctional roles that ArgR shows in the growth of microorganisms, it is considered to be a global regulator in microorganisms and has been used as a target for genetic engineering by researchers (Qu et al. 2020).

Through analysis of the *T. aotearoense* SCUT27 genome, three genes (V518_0585, V518_1870, V518_1864) were annotated as arginine repressor gene. Using homologous recombination, these three genes had been knocked out separately, and mutants of SCUT27/ Δ *argR0585*, SCUT27/ Δ *argR1870*, and SCUT27/ Δ *argR1864* were obtained. Among these mutants, only SCUT27/ Δ *argR1864* exhibited the ideal changes, and the specific activities of xylose isomerase and xylulokinase in SCUT27/ Δ *argR1864* enhanced about 51.52% and 13.90% respectively, compared with that of SCUT27. These results in the increasement of xylose consumption rate. In comparison with SCUT27, it raised by 32.35% under xylose and 76.90% under glucose/xylose mixture. The deletion of *argR1864* not only facilitated sugar metabolism, but also lead to the re-establishment of a new redox balance. Higher cellular concentration of ATP and NAD(H) had positive influence in cell growth and stress response (Wu et al. 2019), which endowed the mutant a better viability of for ethanol production from lignocellulosic hydrolysates. Rice straw, corn cob, and sugarcane bagasse were chosen as the carbon sources in the fermentation broth for ethanol production. The ethanol titer and yield of SCUT27/ Δ *argR1864* was achieved at 5.59, 5.40, 5.24 g/L, and 0.35, 0.37, 0.40 g/g, respectively, which had been greatly promoted about 660.56–760.00% and 344.44–400.00% each (Table 3). These results indicated that knocking out *argR1864* is a convincing strategy to promote the ethanol production by using lignocellulosic hydrolysates (Qu et al. 2020). This research showed that in addition to the relevant genes in the

corresponding metabolic pathways, global regulation genes are also essential to the whole efficiency for ethanol production.

Conclusion and future perspectives

As described as above, *Thermoanaerobacterium* species possess enormous potential to achieve the efficient lignocellulosic biorefinery. The final titers of products, especially ethanol exhibited significant improvement after the metabolic engineering. However, achievement of complex products, such as biobutanol from lignocellulose in one single microbe is still impossible due to the overwhelming metabolic stress. Construction of microbial consortia offers a promising alternative approach, which can reduce metabolic burden through labor division and metabolic cooperation in different microbial species (Jiang et al. 2020a).

Generally, thermophilic microbes exhibit relatively high degradation rates of cellulose owing to its higher growth temperature, which is close to the optimum degradation temperature for cellulase. As the thermophilic bacteria with the capability of hemicellulose degradation capability, *Thermoanaerobacterium* species can take the responsibility of lignocellulose degradation in co-culturing systems. One typical example for butanol production from lignocellulose via CBP was the combination of thermophilic *T. thermosaccharolyticum* M5 and *C. acetobutylicum* NJ4. Among the two-species microbial consortium, *T. thermosaccharolyticum* M5 plays the role in lignocellulose degradation, and the efficient sugars utilization capability can relieve the inhibition to hydrolytic enzymes secreted by strain M5. Additionally, butyrate generated by strain M5 also induced the expression of butanol synthetic genes in strain NJ4. The mutual interaction of these two species exhibited 7.61 g/L of butanol from corn cobs via CBP. While two bottlenecks still present in this microbial consortium. The first one is the limitation of cellulose degradation capability for *T. thermosaccharolyticum* M5 (Jiang et al. 2020b). On the other hand, sequential inoculation was required in the fermentation due to the different temperature demands for these two strains.

To further improve the efficiency of lignocellulosic biorefinery, another well-known anaerobic cellulosic microbe *C. thermocellum* with the efficient cellulose degradation capability is considered as a promising partner for *Thermoanaerobacterium* species. Importantly, *T. saccharolyticum* and *C. thermocellum* show a complementary function to achieve ethanol production from lignocellulose with the similar growth conditions. To eliminate the by-products, organic acids formation can be deleted through genetic modification. Finally, 38 g/L of ethanol could be produced from 92 g/L of crystalline cellulose, achieving nearly

80% of the theoretical maximum concentration (Argyros et al. 2011). The successful example indicates that the suitable chassis microorganisms and combination of complementary functions in synthetic microbial consortia can achieve substantial improvement of lignocellulosic biorefinery. However, until now, the exploitation and application of microbial consortia are still emerging and developing, and the researches were still restricted at the substrate communications, such as fermentable sugars. The insightful and systematical mechanisms of cooperation in microbial consortia still should be explored, which can further guide the design and construction of synthetic microbial consortia.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animals performed by any of the authors.

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