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Review

Biofuel production from lignocellulose via thermophile-based consolidated bioprocessing

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ABSTRACT

The depletion of fossil fuels and their impact on the environment have led to efforts to develop alternative sustainable fuels. While biofuel derived from lignocellulose is considered a sustainable, renewable, and green energy source, enhancing biofuel production and achieving a cost-effective bioconversion of lignocellulose at existing bio-refineries remains a challenge. Consolidated bioprocessing (CBP) using thermophiles can simplify this operation by integrating multiple processes, such as hydrolytic enzyme production, lignocellulose degradation, biofuel fermentation, and product distillation. This paper reviews recent developments in the conversion of lignocellulose to biofuel using thermophile-based CBP. First, advances in thermostable enzyme and thermophilic lignocellulolytic microorganism discovery and development for lignocellulosic biorefinery use are outlined. Then, several thermophilic CBP candidates and thermophilic microbes engineered to drive CBP of lignocellulose are reviewed. CRISPR/Cas-based genome editing tools developed for thermophiles are also highlighted. The potential applications of the Design-Build-Test-Learn (DBTL) synthetic biology strategy for designing and constructing thermophilic CBP hosts are also discussed in detail. Overall, this review illustrates how to develop highly sophisticated thermophilic CBP hosts for use in lignocellulosic biorefinery applications.

1. Introduction

Lignocellulose is the most abundant biomass on earth, with sources including crop residue, such as rice straw, wheat straw, corn stover, and sugar cane bagasse, forestry residues, and municipal solid waste. Statistical data estimate global annual crop residue to be 2445.2 million tons [1,2]. China produces over 1039.5 million tons of crop residues, accounting for more than 42.5% of the global total.

The increasing global demand for energy is significantly depleting fossil fuels, making the diversification of energy sources increasingly important. Biofuel production from lignocellulosic biomass offers a potential solution, as it is considered a sustainable and renewable energy source, and an important means of reducing emissions [3,4]. In recent years, interest in converting lignocellulosic biomass into biofuel via microbial fermentation has grown [5]. However, enhancing processing efficiency remains a technical challenge [6].

Lignocellulose is primarily composed of the major natural polymers, namely cellulose, hemicellulose, and lignin, and other macromolecules [6]. Cellulose and hemicellulose are encased in lignin, a cross-linked phenolic polymer. These combined structures form microfibrils that are resistant to bio-transformation. The complex structure of lignocellulose makes it resistant to enzymatic hydrolysis, reducing biofuel recovery for industrial use.

Recent research has focused on enhancing enzymatic hydrolysis and optimizing the fermentation process [7]. Biofuel production from lignocellulose involves multiple stages, including pretreatment, enzymatic production, saccharification, fermentation, and distillation. Biomass pretreatments include chemical, physical, and biological methods, which are considered effective strategies to reduce lignocellulose recalcitrance [8]. Improving biomass conversion efficiency is crucial for promoting global biofuel energy utilization. Several conversion strategies have been established, including Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF), Simultaneous Saccharification and Co-Fermentation (SSCF), and Consolidated Bioprocessing (CBP) [2]. Among these, CBP has garnered increasing attention due to its ability to achieve hydrolytic enzyme production, lignocellulose degradation, and microbial fermentation in a single step, thereby simplifying the process and reducing costs [9].

An ideal organism for CBP biofuel production from lignocellulose should possess the following characteristics: the ability to produce lignocellulose-degrading enzymes, biomass hydrolysis, and biofuel fermentation. Various organisms, including *Escherichia coli* [10], *Saccha-*

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romyces cerevisiae [11], *Bacillus subtilis* [12], *Zymomonas mobilis* [13], and *Clostridium cellulovorans* [14], have been genetically modified to serve as suitable hosts through metabolic engineering to catalyze all necessary processes under mesophilic conditions.

Compared to mesophilic CBP, the use of thermophiles for the CBP of lignocellulosic biomass offers several techno-economic advantages. These include the elevated efficiency of lignocellulose degradation, minimized risk of contamination, reduced cooling and operation costs, increased saccharification and fermentation rates, and facilitation of downstream product recovery [15,16].

Adapting a microbes to degrade lignocellulose and produce biofuel under extreme conditions is a crucial mechanistic approach. This review discusses recent advances in mechanisms of microbial adaptation to extreme conditions, and the discovery and development of thermostable enzymes and thermophilic microorganisms capable of degrading lignocellulose. It also provides an overview of potential thermophilic candidates for CBP and their characterization. This review also covers genetic modifications of several thermophiles and their potential application in constructing CBP hosts via the synthetic biology strategy of Design-Build-Test-Learn (DBTL).

2. Mechanisms of microbial adaptation to extreme conditions

The adaptation of thermophiles to high temperatures is believed to involve several factors, including cell wall thickness, biosurfactant and/or exopolysaccharide (EPS) production capacity, and heat tolerance of cellular components, including nucleic acids, proteins, and lipids [17,18].

The *Staphylococcus* sp. BSP3 reportedly produces EPS, allowing it to thrive in acidic environments with elevated temperatures characteristic of hot springs [19]. Some genera belonging to Bacillota, including *Geobacillus, Thermoanaerobacterium*, and *Thermoanaerobacter*, possess the capacity to produce biosurfactants [20,21] or EPS [22].

Additionally, the amino acid composition of proteins, the G+C content of genomic DNA sequences, and the lipid composition of membranes enable thermophiles to withstand high-temperature environmental conditions [17]. A novel family of exceptionally long-chain alpha, omega-dicarboxylic acids represents a principal structural fatty acyl component of the membrane lipids of *Thermoanaerobacter ethanolicus* 39E. [23].

3. Thermophilic lignocellulolytic enzymes applied in lignocellulosic biorefineries

The use of microorganisms, particularly thermophilic microbes, in biorefineries that generate lignocellulose-based biofuels and value-added chemicals has gained global attention [16]. Oleaginous microbes can be used to produce biodiesel from lignocellulosic biomass [24]. Thermophilic *Thermoanaerobacterium* species are promising cell factories for lignocellulosic biorefining, as they can directly utilize lignocellulosic materials for biofuel production [15].

The biorefining of lignocellulosic biomass involves enzymatic hydrolysis, saccharification, and other operations with lignocellulose-degrading enzymes playing a crucial role. The use of thermostable enzymes is a key element in lignocellulosic biorefining, which takes place under high-temperature conditions. As a result, lignocellulolytic enzymes from thermophilic bacteria have received increasing attention due to their excellent thermostability. The enzyme systems involved in lignocellulosic biorefining include free enzyme systems, multifunctional megazymes, cellulosome complex systems, and cell-anchored enzyme systems [25]. The utilization of cellulosome complexes to degrade lignocellulosic biomass is a promising approach for biorefineries [26].

In the literature, thermostable lignocellulolytic enzymes including cellulase, xylanase, and laccase, isolated from different environments have been introduced and characterized [27]. For example, *Bacillus stratosphericus* produces an alkaline thermostable laccase that remains

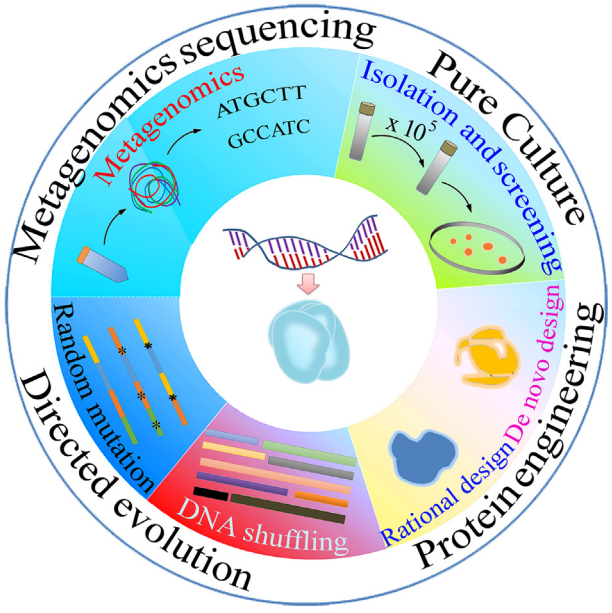

Fig. 1. Design and renovation of lignocellulose-degrading enzymes. To obtain robust enzymes, the strategies for designing and renovating enzymes include the following: 1) Heterologous expression of enzyme genes obtained from metagenomic sequences; 2) Purification of enzymes from pure cultures; 3) Expression of enzyme genes obtained from directed evolution (error-prone PCR or DNA shuffling); 4) Expression of enzyme genes obtained using rational design strategy based on an understanding of the target protein's structures; 5) Expression of enzyme genes achieved through the de novo design of proteins using deep learning-based methods.

stable at 70 °C [28]. Some enzymes from thermophilic archaea can degrade lignocellulosic biomass at higher temperatures, up to around 95 °C [29]. Effective methods for obtaining robust enzymes, including gene editing, genome sequencing, metagenomics, pure culture, site-directed modification, and protein engineering technologies [30-32], are presented in Fig. 1. Deep learning-based methods are becoming increasingly popular for designing proteins [31,32].

Isolating cellulolytic, hemicellulolytic, and solventogenic bacteria is a convenient strategy for achieving direct biofuel production from lignocellulose. Natural ecosystems offer numerous opportunities to screen for microbes that can degrade lignocellulose. Many microbes isolated from unique environments secrete thermostable enzymes capable of degrading lignocellulose.

For instance, *Bacillus licheniformis* isolated from Himalayan soil can produce thermostable cellulase, which achieves optimal activity at 60 °C [33]. Some thermophilic cellulase-producing bacteria, including *Bacillus velezensis* strain MRC 5958 [34] and *Geobacillus* sp. KP43 [35], have been characterized after isolation from hot springs. The strain *Clostridium* SYSU GA15002^T isolated from Yunnan-Tibet hot springs can utilize cellulose, cellobiose, xylan, and untreated straw powder to produce ethanol [36]. A total of 85 glycoside hydrolases (GHs) are encoded in the genome of SYSU GA15002^T. Endoglucanase and β-glucosidase may be responsible for cellulose hydrolysis by SYSU GA15002^T, while endo-1.4-β-xylanases and 1.4-β-xylosidases may be involved in xylan degradation by SYSU GA15002^T. The thermophilic strain *Geobacillus thermodenitrificans* Y7, isolated from chicken manure compost, has an endocellulase gene [37]. A novel cellulose- and xylan-degrading member of the family *Dysgonamonadaceae* has been isolated from a hot spring [38]. A thermophilic cellulose- and hemicellulose-degrading bacterium, *Thermoanaerobacterium* sp. R63, isolated from a tropical dry deciduous forest, exhibits notable cellulase and xylanase activities at 65 °C [39]. A novel hyperthermophilic, anaerobic filamentous archaeon, *Thermofilum adornatum* strain 1910bT, isolated from a terrestrial hot spring, contains

Table 1
Lignocellulose-degrading thermophiles isolated from various environments.

Thermophilic microorganisms	Strains	Sources	Growth temperature (°C)	Substrates	References
Bacteria	*Bacillus licheniformis* PANG L	Himalayan Soil	55	Carboxymethyl cellulose	[33]
	Bacillus velezensis MRC 5958	Natural Hot Spring	50	Rice straw	[34]
	Clostridium thermarum SYSU GA15002^T	Yunnan-Tibet hot springs	45	Cellobiose, Untreated straw powder	[36]
	Geobacillus sp. KP43	Hot Spring Water of Nepal	60	Carboxymethyl cellulose	[35]
	Geobacillus thermodenitrificans Y7	Chicken manure compost	60	Raw switchgrass	[37]
	Seramator thermalis gen. nov., sp. nov. SYSU GA16112^T	Hot spring	45	Cellulose and xylan	[38]
	Thermoanaerobacterium sp. R63	Tropical dry deciduous forest soil	65	Cellulose and hemicellulose	[39]
Archaea	*Thermofilum adornatum* strain 1910b^T	a terrestrial hot spring in Kamchatka, Russia	80	Cellulose	[40]
Fungi	*Aspergillus fumigatus*	Compost and vermicompost	40	Carboxymethyl cellulose	[41]
	Thielavia terrestris LPH172	compost and decaying plant matter	50–60	Rice straw	[42]

Table 2
Protein engineering for the improvement of thermophilic lignocellulolytic enzymes.

Enzymes	Resources	Engineering strategies	Characterizations	Application	References
Endoglucanase Cel6A	*Thermobifida fusca*	Domain engineering	Improvement of the catalytic activity and thermal stability	Plant biomass hydrolysis	[47]
Cellulase Cel5E	*Clostridium thermocellum*	Rational site-directed mutagenesis	Enhancement of specific activity	Biomass conversion	[48]
Xylanase CFXyl3	*Cellulomonas flavigena*	By primary and 3D structure analyses	Improvement of thermostability	Prebiotic xylooligosaccharide manufacturing	[49]
Laccase	Lignin-degrading basidiomycete PM1	Enzyme-directed evolution combined with rational design	Enhancement of the catalytic activity and the thermal tolerance	Kraft pulp bleaching and fibreboard manufacture	[50]
Endoglucanase Cel5	*Stegonsporium opalus*	Structure-guided recombination	Enhancement of thermostability	Industrial bioconversion	[51]
Xylanase XYN10B	*Thermotoga maritima*	Site-directed and error-prone PCR mutagenesis	Improvement of enzyme half-life	Biomass hydrolysis	[52]

four genes encoding four cellulases (Cel25, Cel30, Cel40, and Cel45) [40]. Some thermophilic fungi isolated from compost exhibit lignocellulolytic activity [41,42].

Identified lignocellulose-degrading thermophilic microbes include bacteria, fungi, and archaea. A list of thermophilic microorganisms isolated from various environments within the past 5 years is provided in Table 1. Thermophilic microorganisms that degrade lignocellulose have been isolated from hot springs, compost, soil, and other sources [33-42]. Thermophilic lignocellulolytic archaea can grow at temperatures up to 80 °C. In comparison to the number of lignocellulose-degrading thermophilic bacteria that have been isolated, isolated thermophilic archaea and fungi are relatively scarce (Table 1).

Many extremophile microorganisms cannot be artificially isolated and cultured. Fortunately, the metagenome strategy can be used to obtain new enzymes [43,44]. More importantly, the catalytic activity and stability of enzymes can be improved through directed evolution or rational design [45,46]. Redesigning and constructing glycoside hydrolytic enzymes is essential for enhancing efficiency. Protein engineering can also be employed to enhance enzymes' catalytic activity and thermal tolerance (Table 2). Domain engineering was used to obtain an engineered Cel6A with improved thermostability and enzymatic activity [47]. Rational design was employed to engineer a thermophilic endoglucanase Cel5E from *Clostridium thermocellum*, resulting in improved thermal stability and catalytic activity [48].

Thermophilic lignocellulolytic enzymes have been characterized by various means. However, the degradation of lignocellulose requires the synergistic action of several glycoside hydrolytic enzymes, including cellulase, xylanase, and others. Understanding the mechanism of simultaneous degradation is crucial for improving biomass resource utilization [53]. Prior research has suggested that combining xylanase and cellulase activities results in a pronounced synergistic improvement relative to treatment with cellulase alone in the degradation of mulberry bark [54]. Cellulase activity may be inhibited by xylan and xylooligomers. It has been demonstrated that supplementation with xylanase can relieve inhibition of cellulase activity by xylo-oligosaccharides, as well as facilitate the release of cellulases that were bound to the substrate [55,56].

4. Thermophilic microorganisms as CBP hosts

The conversion of lignocellulosic biomass using lignocellulose-degrading microorganisms and their derived enzymes faces challenges, including enzymatic hydrolysis, fermentation, and product separation [57].

In the traditional bioconversion of lignocellulosic biomass to end products, such as biofuels, lignocellulose is first degraded into simple sugars using hydrolytic enzymes, such as cellulase and xylanase. The filamentous fungus *Trichoderma reesei* has been extensively employed to produce cellulase and xylanase enzymes [58]. These simple sugars are then utilized by efficient ethanol-producing microbes to produce biofuel. Ethanol-producing microbes, including *Saccharomyces cerevisiae* [59], *Z. mobilis* [60], and genetically-modified *E. coli* [61], are capable of fermenting sugars to yield ethanol.

The ability of CBP to improve biomass conversion efficiency is well established. Certain microorganisms, including *C. thermocellum*, can ferment biofuel and efficiently degrade lignocellulose, making them suitable CBP hosts. Thermophilic microorganisms (e.g. *Caldicellulosiruptor bescii*) acting as CBP hosts in this system have attracted increasing attention due to their relatively high efficiency of lignocellulose degradation under thermophilic conditions. More importantly, a high-temperature CBP system using extreme thermophiles shows promise as a strategy for collaborative hydrolysis and fermentation, and for product distilla-

Table 3
Potential CBP candidates for lignocellulose bioconversion in thermophilic processes.

Thermophiles	Growth properties	Characterization	Products	References
Caldicellulosiruptor bescii	Extremely thermophile, optimum temperature of 78 °C	Cellulose and hemicellulose degradation ability; utilizing both C5 and C6 sugars,	Does not natively produce ethanol	[64]
Clostridium thermocellum HSCT2108	Moderate thermophile; optimum temperature of 60 °C	Cellulose degradation ability and acetate as substrates	Short-chain esters of 0.21 g/L	[65]
Geobacillus thermoglucosidasius	Moderate thermophile; optimum temperature of 50–60 °C	Hemicellulose degradation ability Ethanol fermentation ability	Ethanol of 9.04 g/L	[66]
Thermoanaerobacterium aotearoense	Growth temperatures at 55 °C	Utilizing both C5 and C6 sugars; Ethanol fermentation ability	Ethanol of 5.59 g/L	[67]
Thermoanaerobacter BG1L1	Thermophile; optimum temperature of 65 °C	Hemicellulose degradation ability; Ethanol fermentation ability	Ethanol of 0.39–0.42 g/g-sugars	[68]
Thermoanaerobacterium saccharolyticum	Growth temperatures at 30–66 °C	Hemicellulose degradation ability	Ethanol of 61 g/L	[69]

tion [62], which would simplify the process and minimize the effects of ethanol toxicity on cells due to product distillation [63].

To date, suitable candidates for use as hosts of CBP under thermophilic conditions include C. thermocellum, C. bescii, Geobacillus thermoglucosidasius, Thermoanaerobacterium aotearoense, and Thermoanaerobacterium saccharolyticum (Table 3). C. thermocellum is a potential CBP host. It is a native cellulolytic thermophile with an optimum growth temperature of 60 °C. Due to its high cellulose degradation and ethanol fermentation ability, it has been extensively studied as a candidate for a CBP biofuel production platform using lignocellulosic biomass as a substrate [5]. However, the optimum growth temperature for C. thermocellum is around 60 °C, which is well below the boiling point of ethanol.

Members of the genus *Geobacillus* are moderate thermophilic organisms that can grow at optimum temperatures in the range of 50–60 °C. Most of these organisms produce ethanol as a byproduct of their hemicellulose metabolism for growth. *Thermoanaerobacterium* species, with an optimum temperature of about 65 °C, can utilize hemicellulose to produce acetate, lactate, CO_2, H_2, or ethanol. *C. bescii* is an extremely thermophilic cellulolytic bacterium capable of degrading plant biomass at temperatures up to 78–80 °C. However, wild-type *C. bescii* is probably unable to produce ethanol because it lacks genes encoding the enzymes (aldehyde dehydrogenase, Adh, or aldehyde: Fd oxidoreductase, AOR) required for ethanol production [70].

Despite the fact that potential CBP candidates under thermophilic conditions are now available to aid biomass bioconversion, using thermophiles to mediate CBP continues to faces some barriers, including low efficiency. To increase thermophile efficiency in the consolidated bioprocessing of lignocellulosic biomass into biofuel products, several strategies have been established, including the isolation of native single microbes, co-culture with multiple microbes, and the metabolic engineering of microbes [66,67]. Naturally, a single thermophile may not be the most robust chassis for the industrial bio-conversion of biomass into products. Some thermophiles have limitations, including restriction of their sugar metabolizing capability to C6 (6-carbon) sugars, weak cellulose endonuclease activity, or low ethanol yield, while other thermophiles have the ability to utilize both C5 (5-carbon) and C6 sugars. For example, *C. thermocellum* has been under consideration as a suitable host for consolidated thermophilic bioprocessing due to its ability to ferment cellulose, but it cannot ferment C5 sugars, which represent a substantial fraction of the available carbohydrate in lignocellulosic biomass. Co-culturing may thus be required to introduce a broader range of degradation strategies. Co-cultures of three thermophiles, *C. thermocellum, C. stercorarium,* and *T. thermohydrosulfuric*, have recently been shown to improve sugar utilization [71]. Another potential solution is to engineer *C. thermocellum* to simultaneously co-ferment cellulosic and hemicellulosic sugars. The *xylA* gene (encoding for xylose isomerase) and the *xylB* gene (encoding for xylulokinase) from *Thermoanaerobacter ethanolicus* were introduced into *C. thermocellum* DSM 1313 to create an engineered strain able to simultaneously ferment xylose and glucose [72]. Some thermophiles might also play a crucial role in the remediation of environmental contaminants, converting toxic contaminants into useful intermediate products. However, the use of engineered microbes to this end remains to be explored.

5. Engineering thermophilic microorganisms for lignocellulosic biofuel production

Significant improvements in genetic manipulation capabilities have made metabolic pathway engineering of thermophilic microorganisms as chassis cells for biofuel production is a promising strategy for lignocellulosic bioconversion. Thermophiles (e.g. *C. bescii*) with a strong ability to degrade lignocellulosic biomass that do not natively produce biofuel can be engineered to introduce a heterologous gene required for ethanol production [73]. Conversely, some thermophiles with weak lignocellulosic biomass degradation capacity that natively produce biofuel could be engineered to introduce a lignocellulose-degrading enzyme system from other cellulolytic microbes [74,75].

C. thermocellum is an obligately anaerobic, thermophilic Gram-positive bacterium that naturally degrades cellulose using a remarkably effective cellulosome complex system. *C. thermocellum* is also capable of producing both H_2 and ethanol. Thus, *C. thermocellum* has been used extensively for engineering purposes, with the aim of improving ethanol production and increasing tolerance to fermentation inhibitors. For example, the introduction of the *T. saccharolyticum pforA* gene alongside the previously introduced *adhA, nfnAB*, and $adhE^{G544D}$ genes improved ethanol production [76]. The overexpression of the endogenous spermidine synthase in *C. thermocellum* increased resistance to furans and increased ethanol production [77].

To improve the dual utilization of xylose and cellulose, *xylA* and *xylB* genes were integrated into the *C. thermocellum* genome. This engineered *C. thermocellum* strain was capable of co-fermenting cellulose- and hemicellulose-derived sugars simultaneously without carbon catabolite repression [72]. *Thermoanaerobacter* sp. X514 could co-utilize glucose and xylose in parallel without carbon catabolite repression [78]. Interestingly, carbon catabolite repression has been demonstrated in the thermophilic anaerobe *Thermoanaerobacterium saccharolyticum*, which is capable of directly fermenting hexose and pentose [79].

The direct conversion of biomass into desired products represents a novel paradigm for CBP. *G. thermoglucosidasius* has been genetically modified to create a candidate for use in CBP [80]. The dual expression of endoglucanase and exoglucanase in *G. thermoglucosidasius* can enhance the degradation of lignocellulosic material.

In recent years, increased attention has been devoted to the use of metabolically engineered *Caldicellulosiruptor* species as CBP hosts due to their ability to degrade and ferment lignocellulose at temperatures up to 80 °C. Native *C. bescii* cannot produce ethanol because it does not encode acetaldehyde/alcohol dehydrogenase or acetaldehyde dehydrogenase. An ethanol production pathway (AOR-Adh) has been introduced into *C. bescii* cells conferring them with the ability to produce ethanol at 75 °C (close to its boiling point) on the engineered strains [73]. Significant advances have been made in engineering genus *Caldicellulosiruptor* strains

Table 4
Engineering *Caldicellulosiruptor* genus for bioconversion.

Strains	Strategy	Gene manipulation	Remarkable results	References
Caldicellulosiruptor bescii	Heterologous expression of secondary alcohol dehydrogenase (sADH)	Expression using vector	Production of 2,3-butanediol	[81]
Caldicellulosiruptor bescii DSM6725	Deletion of *pyrFA, ldh*, and *cbeI* gene	Gene deletion	Hydrogen	[82]
Caldicellulosiruptor bescii MACB1018	Expression of surface layer homology domain glycoside hydrolase (SLH-GH)	Expression of heterologous gene	Improve its ability to degrade specific plant biomasses	[75]
Caldicellulosiruptor bescii MACB1038	Expression of AOR and adhA; Deletion of lactate dehydrogenase	Gene deletion and expression of heterologous gene	Production of Ethanol	[73]
Caldicellulosiruptor bescii RK87	Expression of thiolase, acetoacetate decarboxylase and CoA transferase	Expression of heterologous gene	Producing acetone and hydrogen	[83]
Caldicellulosiruptor bescii DSMZ6725	Deletion of lactate dehydrogenase and heterologous expression of a bifunctional acetaldehyde/alcohol dehydrogenase	Gene deletion and expression of heterologous gene	Ethanol	[84]

to enhance bioconversion (Table 4). For example, the deletion of lactate dehydrogenase in the engineered strains could increase ethanol production. The introduction of secondary alcohol dehydrogenase (sADH) into *C. bescii* has been shown to produce 2,3-butanediol [81]. The expression of the surface layer homology domain glycoside hydrolase could enhance the capacity of *C. bescii* to degrade specific plant biomass.

6. Current challenges and future prospects in thermophile-based consolidated bioprocessing

The conversion of lignocellulosic biomass to biofuels is gaining increasing attention. However, high costs associated with pre-treatment operations, slow enzymatic hydrolysis, and low ethanol production ultimately increase the overall cost of production. In this respect, high-temperature lignocellulosic conversions offer several advantages, with thermophilic microbes and thermostable enzymes potentially overcome the limitations of existing lignocellulosic biomass conversion processes [85,86]. Despite the promise of thermophilic consolidated bioprocessing, several barriers need to be overcome, and optimizing these processes is crucial for improving cellulosic biofuel conversion efficiency.

6.1. Challenges in improving genetic manipulation techniques for thermophiles

Genetic techniques currently used to generate mutant thermophile strains are mainly based on antibiotic or nutrient selection. Despite the fact that genetic manipulations can aid in mutant strain construction, genetic techniques suitable for thermophilic organisms still face barriers including low efficiency. The exploitation of more powerful genome manipulation tools for thermophilic organisms has thus become a research focus in recent years.

The genetic manipulation of thermophilic organisms is challenging as it is influenced by several key factors, including selection markers, host uptake of DNA, and limited availability of reliable promoters and replicating vectors. The heterologous expression of lignocellulolytic enzymes in thermophilic organisms could improve their ability to degrade biomass [74]. Naturally, suitable promoters are required for heterologous expression. To date, very few inducible promoters from thermophilic bacteria have been characterized. A recent study reported the identification and validation of a strong and inducible promoter from *Thermus thermophiles* [87]. Established GFP-based fluorescent reporter systems for thermophilic bacteria, including *Parageobacillus thermoglucosidasius* and *Thermoanaerobacter kivui*, could expidite genetic manipulation and gene expression quantification [88,89].

Several characterized genes play known roles in ethanol metabolism. For example, ethanol production in *Thermoanaerobacter ethanolicus* is promoted by three alcohol dehydrogenases, AdhA, AdhB, and AdhE [90]. Single gene deletion has identified several genes potentially related to osmotolerance in *C. bescii* [91]. To obtain a robust engineered strain, it is necessary to develop highly efficient genetic tools for manipulating multiple genes. To date, two types of genetic systems have been developed in thermophiles, including gene deletion and integration systems based on antibiotic resistance genes or auxotrophic selection genes, and thermostable Cas protein-based genome editing systems. RNA-mediated adaptive defense systems, termed clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated (Cas) systems (CRISPR/Cas systems), have been developed as powerful tools for gene editing in some representative thermophiles [92-95]. CRISPR/Cas systems for thermophilic genome editing now include a Type Π thermostable Cas9 system, an endogenous Type I-B system, and CRISPR interference (CRISPRi) based on the thermostable inactive Cas9 (dCas9) (Table 5). The establishment of CRISPR/Cas systems for thermophiles has expanded the potential for the manipulation of multiple genes.

6.2. Challenges in constructing robust CBP hosts using a synthetic biology strategy

A thermophile-based ethanol production process offers several advantages, including the co-fermentation of pentose and hexose sugars, reduced risk of microbial contamination, reduced energy input, and easier downstream product recovery [97]. Previous research has shown that aqueous ethanol readily evaporates above 50 °C [98]. Thermophilic strains can further reduce the cost of the process by helping to reduce energy input. However, low ethanol yields and low tolerance to ethanol are typical challenges for thermophilic ethanologists [99]. For example, the hydrolytic capabilities of the thermophilic cellulase from *Geobacillus* sp. R7 are comparable to those of a commercial cellulase, but it produces minimal amounts of ethanol: up to 0.023 g/L using pretreated corn stover as substrate [100].

To obtain a suitable CBP host for biofuel production, the engineering of thermophilic microorganisms is a promising strategy. Current research interests focus on: (1) increasing biofuel production; (2) enhancing tolerance to fermentation inhibitors; (3) improving lignocellulosic degradation. An engineered thermophilic anaerobic bacterium, *Thermoanaerobacterium saccharolyticum* ALK2, could ferment xylan to produce ethanol with high yield [101]. Using the engineered strain (ALK2) in simultaneous hydrolysis and fermentation experiments at 50 °C results in a 2.5-fold reduction in cellulase loading compared with use of *Saccharomyces cerevisiae* at 37 °C [101]. In addition, the volumetric productivity of xylose fermentation to ethanol using engineered strain (ALK2) compares favorably with that of mesophilic strains including recombinant *S. cerevisiae* RWB217 and *E. coli* FBR5 [101].

The rapid development of genetic engineering technologies for thermophiles has facilitated biofuel conversion from lignocellulose via the synthetic biology strategy of DBTL [102]. Many functional elements related to enzymatic hydrolysis, ethanol fermentation, and substrate tolerance have been evaluated. For example, a large number of lignocellulolytic enzymes have been characterized, including cellulolytic enzymes, hemicellulases, and ligninolytic enzymes [29,103]. Some regulatory elements affecting ethanol fermentation have also been identified. Previous reports have shown that the deletion of the *T. saccharolyticum*

Table 5
Genome editing of some representative thermophiles using CRISPR/Cas system.

Thermophiles	Cas proteins	CRISPR/Cas system	Refs.
Clostridium thermocellum	GeoCas9	Type Π	[92]
Thermoanaerobacter ethanolicus	GeoCas9	Type Π	[93]
Thermus thermophilus	CaldoCas9	Type Π	[94]
Clostridium thermocellum	Multiple Cas proteins (such as Cas 3, Cas 4, Cas 5, and Cas8)	Type I-B	[92]
Parageobacillus thermoglucosidasius	Multiple Cas proteins	Type I-B	[95]
Hungateiclostridium thermocellum DSM 1313	Thermostable dCas9	CRISPRi	[96]

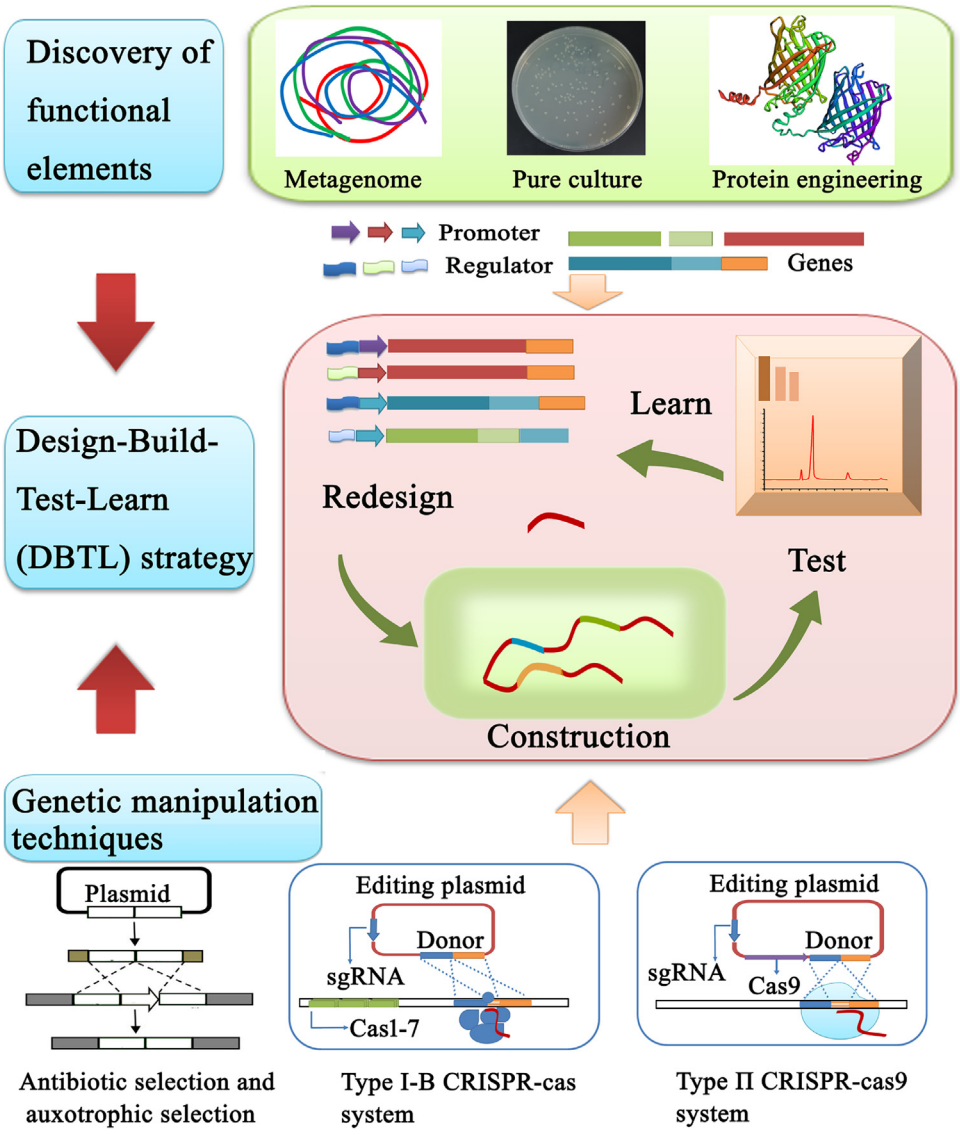

Fig. 2. Synthetic biology approaches to design and construct thermophilic CBP hosts. First, elements such as promoters, regulators and genes are analyzed and reassembled; Second, thermophilic CBP hosts can be created by integrating or deleting functional elements associated with enzymatic hydrolysis or ethanol fermentation using Genetic systems, including gene deletion and integration systems based on antibiotic resistance genes or nutritional selection genes (A) and thermostable Cas proteins-based genome editing systems (B); Third, the functions of the engineered cells were tested; Finally, redesign was performed by understanding the result.

hfsB gene could increase ethanol production [104]. In another example, co-expression with an NADPH-dependent butanol dehydrogenase could reduce sensitivity to inhibitors derived from pretreatment of lignocellulosic biomass [77]. Optimizing and redesigning functional elements are thus important areas for future research. The synthetic biology strategy of DBTL provides a feasible approach to constructing robust CBP hosts (Fig. 2).

6.3. Challenges facing consolidated bioprocessing system optimization

It has been suggested that ethanol production at higher temperatures would facilitate process design [97]. Simultaneous hydrolysis, fermentation, and product separation from untreated waste in a single reactor using thermophilic bacteria has been demonstrated. A techno-economic analysis of a single reactor process using thermophilic bacteria to produce ethanol has shown that the use of a single reactor could reduce the total cost of ethanol production [105].

Thermophile-based CBP for ethanol fermentation can be carried out at high temperatures close to or above ethanol's boiling point. Thermophilic CBP could thus reduce the impact of solvents on the mediating microbe and enable ethanol separation from continuously growing cultures. The engineered *C. bescii* strain can produce ethanol from cellulose at temperatures as high as 83 °C [63]. The thermophilic CBP process for ethanol production from lignocelluloses could be integrated to yield a single-step process. Fig. 3 shows that all steps involved in thermophilic CBP fermentation, including lignocellulolytic enzyme production, lignocellulose hydrolysis, carbohydrate utilization, ethanol fermentation, and product distillation, can be carried out in a single vessel. To opti-

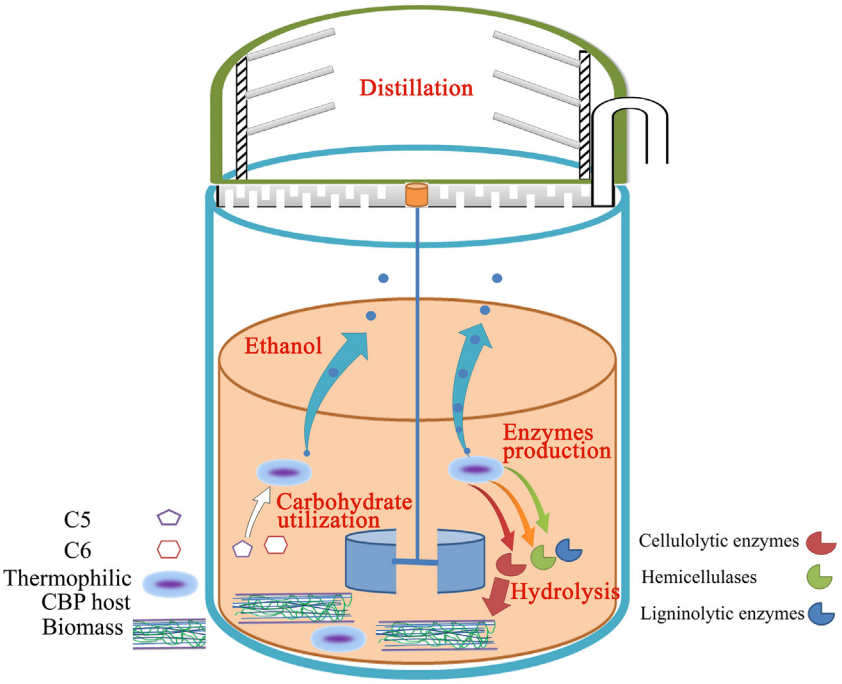

Fig. 3. Lignocellulosic biorefinery using thermophiles as CBP hosts. Enzyme production, hydrolysis, carbohydrate utilization, fermentation, and product distillation were integrated into a single step when thermophiles were used as CBP hosts for lignocellulosic conversion.

mize the process, regulatory parameters, including tank pressure, stirring speed, and temperature control system, must be fine-tuned. A High-Temperature-High-Pressure (HTHP) distillation technique has been applied to obtain ethanol from unprocessed waste using *Thermoanaerobacter mathranii* A3 [105]. Using this HTHP distillation technique, the overall energy retaining efficiency calculated from sugars to ethanol was 1262.7 kJ kg^{-1} dry weight [105]. In this context, the optimization of HTHP technology is expected to improve the efficiency of the distillation process.

7. Conclusions

Biofuel derived from lignocellulose represents a source of sustainable, renewable, and environmentally friendly energy. Thermophilic consolidated bioprocessing offers unique advantages for converting lignocellulose to biofuel. The recent expansion of systems for the genetic manipulation of thermophiles has enabled genetic engineering, and the discovery of functional elements has allowed cells to be redesigned. These advances have created new opportunities to generate biofuels from renewable biomass through thermophile-based consolidated bioprocessing. However, much work remains to be done before thermophilic hosts can be utilized to produce substantial amounts of biofuels or chemicals. This will involve integrating various functional elements, regulating metabolic pathways, and optimizing fermentation processes. Synthetic biology approaches to improve thermophilic microorganisms for use in consolidated bioprocessing are now available to facilitate this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yilin Le: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mengqi Zhang:** Investigation, Formal analysis, Data curation. **Pengju Wu:** Investigation, Formal analysis, Data curation. **Huilei Wang:** Writing – review & editing, Resources, Investigation, Funding acquisition, Formal analysis. **Jinfeng Ni:** Writing – review & editing, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

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