Applied Microbiology and Biotechnology (2024) 108:65
https://doi.org/10.1007/s00253-023-12974-7

MINI-REVIEW

Metabolic engineering of *Caldicellulosiruptor bescii* for hydrogen production

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Received: 7 October 2023 / Revised: 4 December 2023 / Accepted: 15 December 2023

Abstract

Hydrogen is an alternative fuel for transportation vehicles because it is clean, sustainable, and highly flammable. However, the production of hydrogen from lignocellulosic biomass by microorganisms presents challenges. This microbial process involves multiple complex steps, including thermal, chemical, and mechanical treatment of biomass to remove hemicellulose and lignin, as well as enzymatic hydrolysis to solubilize the plant cell walls. These steps not only incur costs but also result in the production of toxic hydrolysates, which inhibit microbial growth. A hyper-thermophilic bacterium of *Caldicellulosiruptor bescii* can produce hydrogen by decomposing and fermenting plant biomass without the need for conventional pretreatment. It is considered as a consolidated bioprocessing (CBP) microorganism. This review summarizes the basic scientific knowledge and hydrogen-producing capacity of *C. bescii*. Its genetic system and metabolic engineering strategies to improve hydrogen production are also discussed.

Key points

- *Hydrogen is an alternative and eco-friendly fuel.*
- *Caldicellulosiruptor bescii produces hydrogen with a high yield in nature.*
- *Metabolic engineering can make C. bescii to improve hydrogen production.*

Keywords Hydrogen · Lignocellulosic biomass · *Caldicellulosiruptor bescii* · Consolidated bioprocessing (CBP) · Metabolic engineering

Introduction

Currently, there are increasing concerns about serious environmental problems such as the greenhouse effect, global climate change, fine dust caused by the use of fossil fuels, and other complications caused by the depletion of fossil fuels (Fawzy et al. 2020; Manisalidis et al. 2020; Martins et al. 2019). To address these issues, there has been a growing interest in biofuels, such as bioethanol, biodiesel, and biohydrogen, which are produced through biological processes using various renewable resources (Cha et al. 2013a; Hoang et al. 2023; Jeswani et al. 2020; Martínez-Jaramillo et al. 2019). Among these biofuels, hydrogen is an attractive and promising option for two important reasons: (i) hydrogen is non-toxic and does not release the greenhouse gas CO_2 when combusted (clean energy), and (ii) it carries higher energy compared to other hydrocarbon fuels (Hassan et al. 2023; Okolie et al. 2021). Therefore, hydrogen has been suggested as a major chemical energy carrier and could

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Published online: 09 January 2024

be utilized as a high-energy storage for transportation vehicles (Miller et al. 2021). Hydrogen can be utilized not only as a commercial transportation fuel but also in the chemical industry (chemical looping hydrogen) for the production of methanol and ammonia (Palone et al. 2023), as well as in various other industries such as electronics, metals, and food. Unfortunately, hydrogen does not exist in a free form in nature. However, it is present in water or in the main components of all living organisms, suggesting that hydrogen can be produced through biological processes (Akhlaghi and Najafpour-Darzi 2020; Lepage et al. 2021).

The biological production of hydrogen can be accomplished through various steps including enzymatic saccharification of renewable biomass to convert into fermentable sugars, as well as anaerobic fermentation of these sugars to hydrogen by anaerobic bacteria (Fig. 1) (Alicia Benitez et al. 2021; Cha et al. 2013a, 2016). C5 and C6 sugars, which are derived from a variety of carbohydrates like glucose and xylose found in plant biomass, are oxidized via the Embden–Meyerhof–Parnas glycolytic pathway (Fig. 1) to produce acetate, lactate, carbon dioxide, and hydrogen (Cha et al. 2013a, 2016, 2023; Chandel 2021). In terms of final fermentative products, carbon flow is directed towards lactate or acetyl-CoA, while electrons flux towards lactate and H_2 from pyruvate, which serves as a major metabolic branch point (Fig. 1).

Microorganisms are crucial for achieving high yields of hydrogen, especially with the involvement of thermophiles such as *Thermoanaerobacter tengcongensis* (~4.0 mol H_2/mol glucose) (Soboh et al. 2004), *Thermotoga maritima* (~4.0 mol H_2/mol glucose) (Schroder et al. 1994; Singh et al. 2019), and *Thermococcus kodakarensis* (~3.3 mol H_2/mol glucose) (Burkhart et al. 2019; Kanai et al. 2005). The hyperthermophile, *Pyrococcus furiosus* (optimal temperature 90 °C), also produced ~2.8 mol H_2/mol glucose (Servé and Kengen 1994; Song et al. 2019) although it is smaller compared to others. The utilization of high temperatures (> 50 °C) should be beneficial for hydrogen production due to reduced viscosity, improved mixing, trace contamination, enhanced reaction rates, and the elimination of the need for reactor cooling (Shahbeik et al. 2022). Additional microorganisms capable of producing hydrogen are listed in Table 1. While most hydrogen-producing microorganisms rely on starch-based biomass, which can be easily saccharified, the direct production of hydrogen from lignocellulosic biomass is challenging for microorganisms as it requires additional steps, such as pretreatment, enzymatic saccharification, and the generation of fermentation inhibitors (Zafar et al. 2021). Therefore, the development of microbial strains that fermenting lignocellulosic biomass effectively is necessary (Kim et al. 2022).

The genus *Caldicellulosiruptor* is a thermophilic microorganism with cellulosic activity. It can produce hydrogen at high rates from lignocellulosic biomass, with an optimal growth temperature between 75 and 80 °C (Scott et al. 2019). *C. bescii* can serve as a consolidated bioprocessing (CBP) organism (Fig. 2) because it can utilize both C5 and C6 sugars simultaneously and directly convert lignocellulosic biomass without conventional pretreatment steps (Fig. 2) (Cha et al. 2013a, 2016; Chung et al. 2014; Periyasamy et al. 2023). The *C. bescii* genome encodes many carbohydrate-active enzymes (CAZymes), which are multi-domain enzymes with cellulolytic and hemicellulolytic activity and utilize a broad range of substrates,

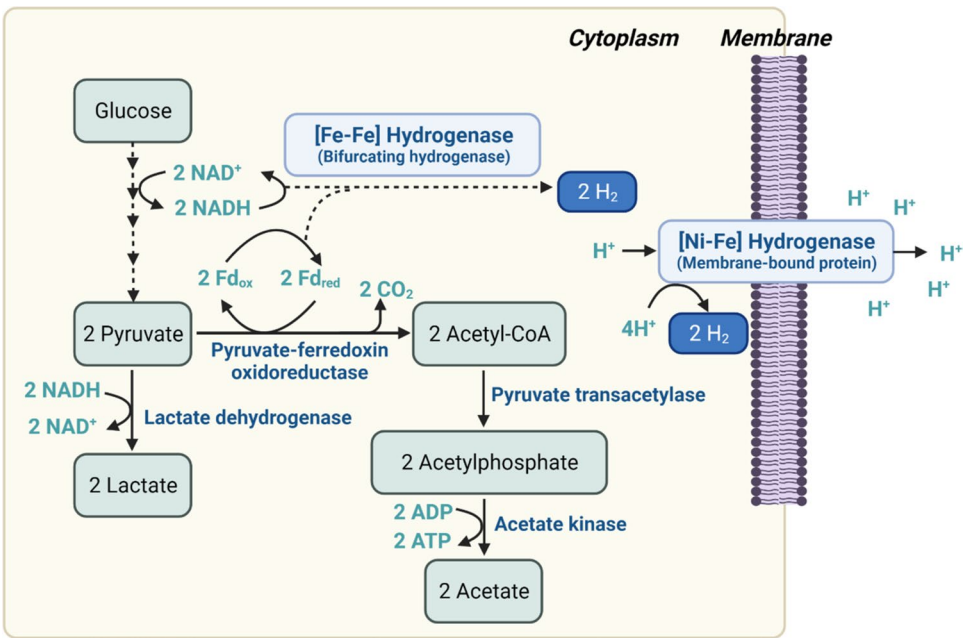

Fig. 1 A predicted and simplified biosynthetic pathway for hydrogen production in *C. bescii*

Table 1 Hydrogen production by various microorganisms

Microorganisms	Optimal growth temp. (°C)	$Y_{H/G}$ (mol/mol)*	Final products (except for H_2 and CO_2)	References
Clostridium acetobutylicum ATCC824	30	1.79	Acetate, acetone, butanol, butyrate	Oh et al. (2009)
Clostridium beijerinckii	37	2.81	—-	Lin et al. (2007)
Clostridium butyricum	37	2.29	—-	Lin et al. (2007)
Clostridium thermosaccharolyticum LMG 6564	55	1.6	Acetate, lactate, ethanol, butanol, butyrate	Vancanneyt et al. (1990)
Clostridium thermocellum 27,405	60	1.6	Acetate, lactate, ethanol, formate	David et al. (2006)
Caldicellulosiruptor saccharolyticus	70	2.5	Acetate, lactate	de Vrije et al. (2007)
Caldicellulosiruptor bescii JWCB001	75	1.8	Acetate, lactate	Cha et al. (2013a)
Escherichia coli	37	1.4	Ethanol, acetate, lactate, succinate	Seppälä et al. (2011)
Escherichia coli MG1655	37	0.56	Acetate, lactate	Cofré et al. (2016)
Enterobacter aerogenes	37–55	1.92	Acetate, lactate, ethanol	Jayasinghearachchi et al. (2009)
Enterobacter aerogenes E.82005	38	1.0	Acetate, lactate, ethanol	Tanisho (1998)
Klebsiella oxytoca	38	1.0	—-	Minnan et al. (2005)
Klebsiella pneumoniae	37	2.7	Acetate, lactate, formate, 2,3-butanediol, ethanol	Niu et al. (2010)
Thermoanaerobacter tengcongensis JCM 11007	75	4.0	Acetate	Soboh et al. (2004)
Thermotoga maritima DSM 3109	80	4.0	Acetate	Schroder et al. (1994)
Thermococcus kodakarensis KOD1	85	3.3	Acetate, alanine	Kanai et al. (2005)
Thermotoga neapolitana	77	2.4	—-	de Vrije et al. (2010)
Thermoanaerobacterium thermosaccharolyticum	55–60	1.8	Ethanol, D-/L-lactate, acetate	Liu et al. (2008)
Pyrococcus furiosus DSM 3638	90	2.8	Acetate, alanine	Servé and Kengen (1994)

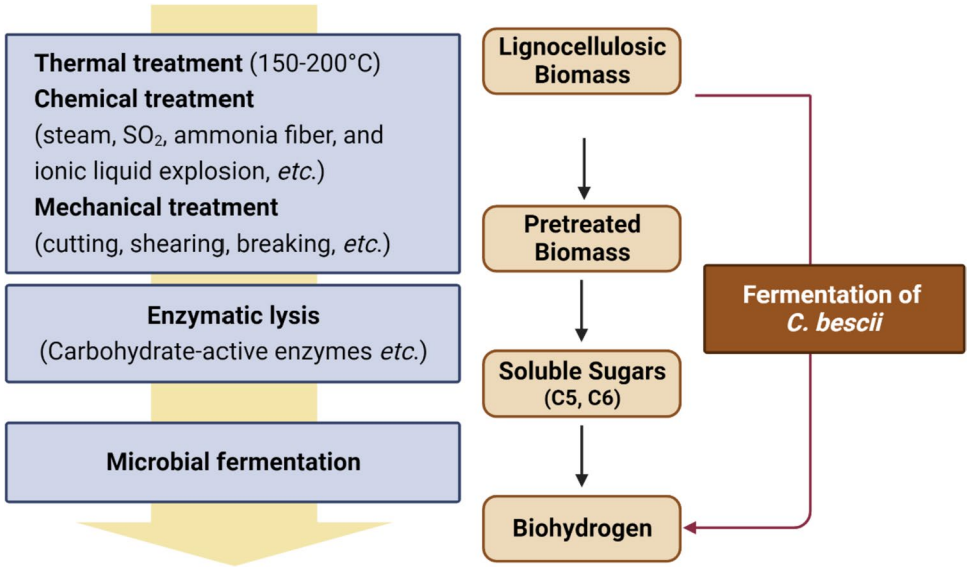

Fig. 2 Hydrogen production with no or reduced pretreatment by a CBP (consolidated bioprocessing) organism, *Caldicellulosiruptor bescii*

including plant biomass, without the need for conventional pretreatment (Kim et al. 2019). Therefore, there is potential to improve the economics of biofuel production from lignocellulosic biomass by skipping thermal, chemical, and mechanical treatment steps (Cha et al. 2013a; Chung et al. 2014).

This review provides the scientific knowledge and data on *C. bescii*, focusing on its H_2 production capacity, genetic

system, and metabolic engineering strategies, which can make *C. bescii* a highly efficient organism for hydrogen production at high temperatures.

Biosynthetic pathway of hydrogen of *C. bescii*

Usually, the genus *Caldicellulosiruptor* produces relatively high yields of H_2 (4 mol of H_2/ mol of glucose) compared with other microorganisms (Cha et al. 2013a, 2016; Straub et al. 2020). Additionally, acetate should be coupled with H_2 production for the reoxidation of NADH (a two-electron donor) and ferredoxin (a one-electron donor) (Buckel 2021; Cha et al. 2016; White 2012) (Fig. 1).

As shown in Fig. 1, H2 is produced through proton reduction catalyzed by hydrogenases (Cha et al. 2016; Jay et al. 2020; Lu and Koo 2019). These hydrogenases are metalloenzymes that contain iron in their active site, such as di-iron, nickel–iron, or iron-sulfur clusters (Lu and Koo 2019). Specifically, *Caldicellulosiruptor* spp. have only two types of hydrogenases: bifurcating [Fe–Fe] hydrogenase and [Ni–Fe] hydrogenase (Cha et al. 2013a, 2016; Zhang et al. 2021). NADH and ferredoxin are catabolized by the bifurcating [Fe–Fe] hydrogenase, resulting in the production of H_2 (Cha et al. 2016; Zhang et al. 2021). On the other hand, the [Ni–Fe] hydrogenase is a membrane-bound heterodimer and is widely found in nature (Alfano and Cavazza 2020). Although the [Ni–Fe] hydrogenase also catalyzes H_2 production, the bifurcating [Fe–Fe] hydrogenase is the primary enzyme for H_2 production in *C. bescii,* while the main role of the [Ni–Fe] hydrogenase is to pump out protons across the cellular membrane to generate the "proton motive force" (Kaila and Wikstrom 2021; White 2012).

Hydrogen production from C5 and C6 sugars

In most of the studies reported, the maximum amount of hydrogen produced was 2.0–3.8 mol of H_2 /mol of glucose (C6 sugars) due to the formation of co-products such as lactic acid and acetic acid (Esercizio et al. 2021). The theoretical molar yield of hydrogen from xylose (C5 sugar) fermentation is 3.3 mol of H_2/mol of xylose (C5 sugar) with acetate as the sole byproduct, but the reported values were lower than 2 mol of H_2/mol of xylose (Chiu-Yue Lin and Hung 2008). Hydrogen production has been reported to be between 0.5 and 4 L/L/day (Ghimire et al. 2015; Beckers et al. 2015). The nature, carbohydrate content, and biodegradability of carbon substrate play an important role in the H_2 yield, production rate, and overall economics of the process (Nanqi et al. 2011). Many bacterial species have been reported to produce hydrogen from C5 and C6 sugars, including enteric bacteria such as *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Escherichia coli*, which produce about 1–2 mol of H_2/mol of glucose (Yoshida et al. 2006). *Clostridium* spp. also produce similar amounts (Liu et al. 2006). *Caldicellulosiruptor* spp. produce about 3–4 mol of H_2/mol of glucose (Willquist et al. 2010). *Enterobacter* utilizes a formate-H_2 lyase, and *Clostridium* spp. use a ferredoxin-dependent hydrogenase to avoid the thermodynamically unfavorable formation of H_2 from NADH (Schut and Adams 2009). Based on previous studies on various microorganisms, metabolic engineering and pre-treatment of substrates can increase hydrogen production by improving the biodegradability of substrates. In summary, *C. bescii* can be a superior organism as it does not contain competing pathways other than lactate, offers the potential to produce maximum amounts of H_2 (4 mol of H_2/mol of C5 and C6 sugars), and is tractable to metabolic engineering.

Hydrogen production from various biomass by *C. bescii*

The most important aspect of *C. bescii* is its ability to decompose various monosaccharides and polysaccharides, such as glucose, xylose, crystalline cellulose, and non-pretreated plant biomass. To compare hydrogen production from real-world substrates, *C. bescii* wild type (JWCB001) and its mutant strains (JWCB005 and 017) were grown on 0.5% switchgrass (Cha et al. 2013a). The strains were incubated for 120 h; then, hydrogen production was measured. The hydrogen productions of JWCB001 and JWCB005 were ~ 1.8 mol/mol of glucose and ~ 1.7 mol/mol of glucose, and it is a bit lower than H_2 production by *Caldicellulosiruptor saccharolyticus* (~ 2.5 mol/mol of glucose). However, in the case of *C. saccharolyticus*, yeast extracts were added to the growth medium. Even without the addition of yeast extracts, the engineered *C. bescii* strain JWCB017 produced significantly more hydrogen (~ 3.4 mol/mol of glucose; Table 2) (Cha et al. 2013a). Actually, *C. bescii* lacks the enzyme required for ethanol production. Chung et al. reported the heterologous expression of *adh*E to enable *C. bescii* to produce alcohol (Chung et al. 2014). However, this resulted in reduced hydrogen production as carbon and electrons were redirected for alcohol production (Chung et al. 2014). *C. bescii* can also produce hydrogen from barley straw and *Miscanthus*. In a previous study, JWCB018 without the chromosomal *ldh* gene produced 63% and 25% more hydrogen from barley straw and *Miscanthus* than the wild-type strain (JWCB001), respectively. It might be due to a decrease in lactate production by interrupting lactate dehydrogenase function by a native active transposon (Cha et al. 2023, 2013b). Yilmazel and Duran reported hydrogen production in co-substrate reactors, where *C. bescii* was grown on four different substrate mixtures of cattle manure (CM), switchgrass (SG), and biosolid (BS). *C. bescii* grown on BS + SG + CM (~ 15.0 mM) showed much better hydrogen production compared to BS + SG (~ 11.0 mM), exhibiting

Table 2 Hydrogen production from biomass by *C. bescii*

Strain	Substrate	Hydrogen produced	References
C. bescii DSM 6725 (wild type)	CM + SG	~ 11.5 mM	Yilmazel and Duran (2021)
C. bescii DSM 6725 (wild type)	CM + BS	~ 13.0 mM	
C. bescii DSM 6725 (wild type)	SG + BS	~ 11.0 mM	
C. bescii DSM 6725 (wild type)	CM + SG + BS	~ 15.0 mM	
C. bescii DSM 6725 (wild type)	0.5% SG	~ 1.8 mol/mol of glucose	Cha et al. (2013a)
C. bescii JWCB005 (*ΔpyrAF*)	0.5% SG	~ 1.7 mol/mol of glucose	
C. bescii JWCB017 (*ΔpyrAF Δldh*)	0.5% SG	~ 3.4 mol/mol of glucose	
C. bescii JWCB018 (*ΔpyrAF Δldh ΔcbeI*)	1% Barley Straw	~ 12.0 mM	Cha et al. (2023)
C. bescii JWCB018 (*ΔpyrAF Δldh ΔcbeI*)	1% Miscanthus	~ 17.0 mM	
C. bescii JWCB018 (*ΔpyrAF Δldh ΔcbeI*)	2% SG	14.5 mM	Chung et al. (2014)
C. bescii JWBC032 (*ΔpyrAF ldh::ISCbe4 Δcbe1::PS-layer Cthe-adhE2/(ura-/5-FOA^R)*)	2% SG	9.8 mM	
C. bescii JWCB038 (*ΔpyrFA Δldh CIS1::PS-layer Cthe-adhE ΔhypADFCDE/(ura-/5-FOA^R)*)	2% SG	~ 4.0 mM	Cha et al. (2016)

CM cattle manure, *SG* switchgrass, *BS* biosolids

synergistic effects of co-fermentation of these feedstocks (Yilmazel and Duran 2021).

Genetic system for *C. bescii*

There are many interesting thermophiles that produce interesting and important chemicals. The ability to manipulate *C. bescii* genes is required to make the hyper-thermophilic strain more useful in the real world. However, the wild-type strain of *C. bescii* is not sufficient to produce biofuel, so it needs to be metabolically engineered to produce biofuel at a suitable yield. One of the most difficult aspects of studying hyperthermophiles like *C. bescii* is the lack of genetic tools for metabolic engineering. In order to develop a genetic tool, there are several requirements: (i) overcoming the restriction-modification (R-M) system, (ii) constructing a *E. coli-C. bescii* shuttle vector, (iii) establishing a transformation method, and (iv) selecting a selection marker (Chung et al. 2013a). One significant barrier to develop genetic tools for uncharacterized microorganisms, especially hyperthermophiles, is the lack of selectable markers. Antibiotics are typically used in mesophilic bacteria, but not in thermophiles because thermostable antibiotic markers are usually not available at high temperatures over 70 °C (Crosby et al. 2019). Because of their high growth temperatures, the genetics of most thermophiles depend on auxotrophic mutant strains. This method is often used for many thermophiles including *Caldicellulosiruptor* (Cha et al. 2013a, 2016; Chung et al. 2014; Lipscomb et al. 2016), *Sulfolobus* (Wagner et al. 2012; Zheng et al. 2012), and *Thermotoga* sp. RQ7 (Han and Xu 2017). The selection method (using an auxotroph mutant strain) for transformation in *C. bescii* relies on the loss of the uracil biosynthetic enzyme coding for orotidine-5′-monophosphate (OMP) decarboxylase (*pyr*F), which was first described in yeast (Boeke et al. 1984) and has been a useful genetic tool in both bacteria and archaea (Lucas et al. 2002). In order to generate a spontaneous *pyr*F mutant strain, the cells were grown on low osmolarity-defined growth medium (LOD) (Farkas et al. 2013) supplemented with uracil and 5-fluoroorotic acid (5-FOA). The strain with *ΔpyrFA*, *C. bescii* JWCB005, was obtained as a host strain for gene manipulation (Chung et al. 2013a). In order to create a shuttle vector capable of replicating in both *Escherichia coli* and *C. bescii*, the *pyr*F gene for uracil auxotroph was cloned and inserted into pBAS2 vector (Clausen et al. 2004), which is a small plasmid with a replication origin of the two plasmids in *C. bescii*. The *E. coli/C. bescii* shuttle vector pDCW89 was constructed by linking a low copy replication origin of *E. coli*, PSC101, and apramycin-resistant gene cassette (Apr^R) to pBAS2 vector (Clausen et al. 2004; Dam et al. 2011). Although a shuttle plasmid is available, there is still another barrier that needs to be addressed to manipulate *C. bescii* genes. The biggest obstacle when transforming foreign DNA for deletion/insertion of a gene is the restriction-modification (R-M) system, which recognizes the difference in DNA methylation when foreign DNA is introduced into the cells, leading to the degradation of the foreign DNA by the restriction system in the strain (Chung et al. 2013b). When the pDCW89 shuttle vector is transformed into the *pyr*F deleted strain by electroporation, the transformation competency is significantly low because *C. bescii* has its own restriction endonuclease, CbeI (Cbe_2438), which was discovered by Chung et al. (2011). CbeI has a HaeIII-like activity and is a type II restriction endonuclease that cleaves unmethylated sequences at 5′-GG/CC-3′ (Chung et al. 2013b; Han et al. 2014). The CbeI

activity should be removed in the host strains for successful DNA transformation. CbeI (Cbes_2438) and a neighboring α-class N4-cytosine methyltransferase (M.CbeI, Cbes_2437) were confirmed to be the counterpart of the R-M system in *C. bescii* (Chung et al. 2013b). Treatment of the *E. coli*/C. *bescii* shuttle plasmid DNA with cloned M.CbeI protein resulted in efficient transformation. Chung et al. also reported a *cbeI* deletion (Cbes_2438) and generated a new host strain, *C. bescii* JWCB018 (Δ*pyrAF* Δ*cbeI* Δ*ldh*), through homologous recombination. JWCB018 (Δ*pyrAF* Δ*cbeI* Δ*ldh*) can be transformed by DNA isolated from *E. coli* without in vitro methylation (Chung et al. 2013b). A brief procedure of gene deletion is described in Fig. 3.

Enhanced hydrogen production by metabolically engineered *C. bescii*

The ultimate goal of biofuel production is to construct a microbial strain for a consolidated bioprocessing (CBP) organism (Olguin-Maciel et al. 2020), which is an organism capable of producing biofuels, such as alcohol and hydrogen gas, through a one-step process using plant biomass (Fig. 2).

Previous research has shown (Cha et al. 2013a, 2016, 2023; Farkas et al. 2013) that various stains of *C. bescii* were constructed using metabolic engineering techniques to enhance its capabilities as a CBP organism (Cha et al. 2013a). The first engineered *C. bescii* strain, obtained through a newly developed metabolic engineering technique, was a mutant strain with a deletion in the lactate dehydrogenase gene (*ldh*), resulting in the complete absence of lactate production. By removing the lactate production pathway, more carbon should be flowed to acetate and more electron flux carried by NADHs runs to acetate and hydrogen production from pyruvate. The *ldh*-deleted strain of *C. bescii* (JWBC017) was grown on 0.5% switchgrass supplemented as the sole carbon source and showed increased production of acetate and hydrogen but no lactate production (Fig. 1) (Cha et al. 2013a). The *ldh*-deleted strain producing no lactate showed a 6.5% conversion of cellobiose to acetate (9.2 mM) with 105% overall carbon recovery (Cha et al. 2013a). The metabolically engineered strain JWCB017 (*ΔpyrFA, Δldh*) produced more hydrogen (~ 3.4 mol/mol of glucose) compared to wild-type *C. bescii* (~ 1.8 mol/mol of glucose) and *C. saccharolyticus* (~ 2.5 mol/mol of glucose) (de Vrije et al. 2007). This indicates that *C. saccharolyticus* wild type produced more hydrogen than *C. bescii* wild type due to the use of yeast extract in the culture media, which can improve growth and yield.

As mentioned earlier, hydrogenases play a key role in microbial energy metabolism, but the exact nature and function of these enzymes remain unclear. Cha et al. (2016) reported the deletion of a gene cluster called *hypABFCDE*, which encodes the maturation proteins for the *C. bescii* [Ni–Fe] hydrogenase. The resulting mutant strain, JWCB038 (Cha et al. 2016), exhibited slower growth compared to its wild type or parent strain (JWCB005, *ΔpyrFA*) because the main function of the [Ni–Fe] hydrogenase may act as a proton pump generating a proton motive force (PMF) across the cellular membrane for ATP synthesis (Fig. 1). The data (Cha et al. 2016) also indicated that the mutant strain JWCB038 did not exhibit a significant reduction in hydrogen production, suggesting that the [Ni–Fe] hydrogenase may not be the main enzyme involved, and that the bifurcating [Fe–Fe] hydrogenase might be the primary enzyme responsible for hydrogen production in *C. bescii*.

To remove the R-M system in *C. bescii* to facilitate metabolic engineering, a *cbe*I deletion strain was generated (Chung et al. 2013b). However, the function of LDH was also disrupted by a native active transposon at the same time (Cha et al. 2013b). The resulting strain JWCB018 (*ΔpyrFA*

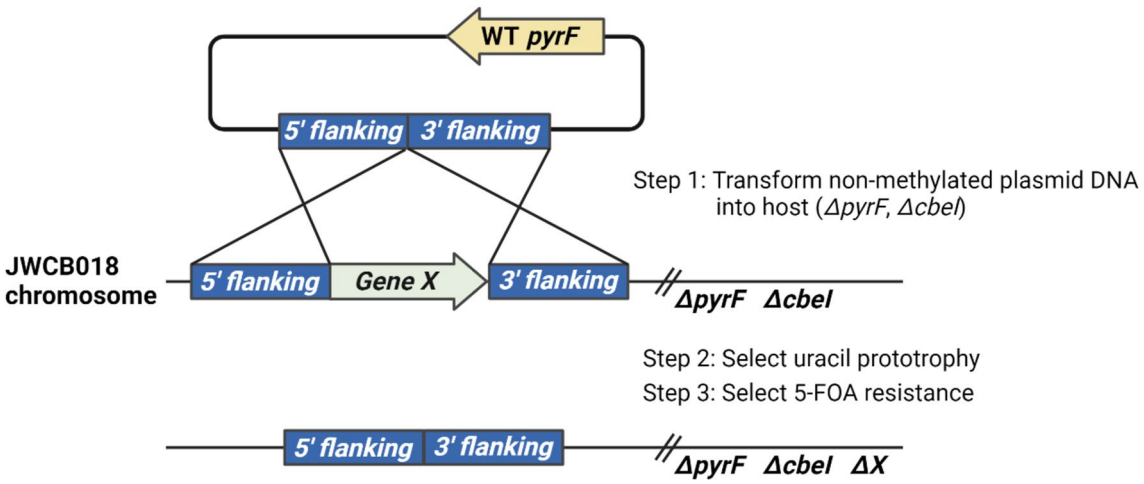

Fig. 3 A procedure for editing a target gene in the genome of *C. bescii*

ΔcbeI Δldh) (Cha et al. 2013b; Chung et al. 2013b) was grown on 10 g/L cellobiose, 20 g/L Avicel, 10 g/L barley straw, and 10 g/L *Miscanthus* as carbon sources and then compared to the *C. bescii* wild type for hydrogen production (Cha et al. 2023). Because of the interruption of the lactate producing pathway in the mutant strain JWCB018, there was an increase in NADHs carrying electrons, resulting in enhanced H_2 production. This study showed that JWCB018 exhibited up to 25%, 21%, 33%, and 25% increases in H_2 production on 1.0% cellobiose, 2% Avicel, 1.0% barley straw, and 1.0% *Miscanthus*, respectively (Cha et al. 2023). These findings clearly indicate that appropriate metabolic engineering can significantly enhance the production of H_2 and other valuable chemicals.

Further strategies to improve hydrogen production by *C. bescii*

Although very useful gene manipulation techniques for *C. bescii* metabolic engineering have been developed and research for biofuel production is being intensively conducted, there is still a need for the development of more efficient genetic tools and techniques for thermophiles, especially *C. bescii*. For example, promoters for high expression and better replicating plasmids for heterologous gene expression should be developed. Instead of using an uracil auxotroph, new thermo-stable antibiotic screening techniques, such as thermo-stable kanamycin (Lipscomb et al. 2016), will also be needed to save time and effort. However, the current methods for deletion and insertion of multiple genes are sufficient.

There is one possible strategy to increase hydrogen production from real-world plant biomass. This strategy involves utilizing the genetic tools and the techniques developed for *C. bescii*. The first step is to remove the pathway for acetate production by deleting the phosphate acetyltransferase-encoding gene (*pta*, Cbes_1494). By deleting *pta*, the electrons carried by ferredoxins can be used by both types of hydrogenases such as [Fe–Fe] hydrogenases and [Ni–Fe] hydrogenases, resulting in more hydrogen production and ATP synthesis. Another potential strategy involves the manipulating of various glycosyl hydrolases by encoding genes by overexpressing the corresponding genes and manipulating regulatory genes to increase the decomposing efficiency of unpretreated plant biomass.

Conclusions

In this review, we highlighted that *C. bescii* can produce hydrogen directly from plant biomass without conventional pretreatment processes. Additionally, novel efficient methods for genetic modification of *C. bescii* have been developed through the deletion of *cbeI*, which is a thermostable type II restriction endonuclease. Overall, previous studies have demonstrated that *C. bescii* can be metabolically engineered to enhance hydrogen production. These would help *C. bescii* to efficiently produce hydrogen from biomass and biowaste including lignocellulosic biomass, cattle manure, and wastewater sludge (Table 2).

Author contribution M Cha and JK Kim surveyed the literature and wrote the manuscript. SJ Kim and M Cha designed the structure of the manuscript. WH Lee, H song, TG Lee, and SK Kim reviewed and revised the mini-review. All authors read and approved the manuscript.

Funding This study was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) and Korea Smart Farm R&D Foundation (KosFarm) through the Smart Farm Innovation Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA), MSIT, and Rural Development Administration (RDA) (No. 421045–03). Also, this research was carried out with the support of the Cooperative Research Program for Agriculture Science and Technology Development (PJ01708203) through the Rural Development Administration and the "Regional Innovation Strategy (RIS)" through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (MOE) (2021RIS-002). This research was also supported by the Chung-Ang University Graduate Research Scholarship (Academic Scholarship for the College of Biotechnology and Natural Resources) in 2023.

Declarations

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

Conflict of interest The authors declare no competing interests.

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