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Paper

Direct hydrogen production from lignocellulose by the newly isolated Thermoanaerobacterium thermosaccharolyticum strain DD32 †

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Tao Sheng[a], Lingfang Gao[b], Lei Zhao[a], Wenzong Liu[b], Aijie Wang[a.b]*

Biofuel from lignocellulosic materials is a promising type of fuel because it does not compete with food supplies and has asustainable production process. However, the primary obstacle for producing biofuels from lignocellulose is the low-energy productivity ofmicroorganisms. In this research, thermophilic bacterial strain DD32, which effectively converts lignocellulose to hydrogen, was isolated and characterized. This strain was identified asThermoanaerobacterium thermosaccharolyticum according to 16S rRNA sequence homology. The maximumH2production yield reached 12.08mmol H2 g−1 Avicel, which is equivalent to 2.17 mol H2mol−1 glucose, at the optimal condition of 55 °C and pH 7.5 with 5.0 gL−1Avicel. To our knowledge, this resultrepresents the highest H2 yield from cellulose for thermophilic bacterial monocultures reported so far. Moreover, the hydrogen productivity of strain DD32 from raw (non-pretreated) lignocellulosic biomass is also tested. Results show that the highest hydrogen yield and lignocelluloses degradation rate reached 6.38mmol H2 g−1 and 44.29% from corn stalk after 72-h of incubation.This yieldwas almost 2–3 times thatof other thermophilic strains. These results suggested that newly isolateT. thermosaccharolyticum DD32 could serve as an effective microbial catalyst for lignocellulosic hydrogen production.

Introduction

Energy crises and global warming are considered severe global problems.[1]According to one study, the Earth's oil reserves could run out during this century.[2] However, until now, most countries still usefossil fuels as their first choice;thus, the only possible solution to this crisis is to find a sustainable (renewable) and economically feasible source of alternative energy.[3]Bio-hydrogen is a type of biofuel that is considered an important option as an alternative energy source to conventional petroleum because of its high calorific value and non-generation ofharmful combustion by-products.[4-6] Bio-hydrogen has already been produced from lignocellulose on a large scale worldwide.[7]

Lignocellulose resources refer to the organisms produced by photosynthesis and crop, agricultural waste, and wood waste decomposition. Biofuels constitute 14% of global primary energy and is the fourth largest energy source following coal, oil, and natural gas.[8]Bio-hydrogenfrom lignocellulose can meet the current energy demand and mitigate climate change[9] to achieve a sustainable environment.[10]

The greatest challenge to microorganisms in converting lignocellulose into bio-hydrogen is its highly polymeric and stable structure; hence, prior to hydrogen fermentation, lignocellulose must be hydrolyzed into oligosaccharide, which is preferredby most hydrogen-producing microorganisms.Therefore, lignocelluloses should be broken downinto oligosaccharidesby hydrolysis before bio-hydrogen production.At present, the methods of lignocelluloses hydrolysis focus on dilute acid/alkali or cellulase. However, lignocelluloses hydrolysishas obvious disadvantages, such as relatively high cost, high equipment requirements[11], generation of inhibitory by-products in chemical hydrolysis process, and time-consuming biological pretreatment, thusundoubtedly limiting the use of lignocellulose on bio-hydrogen.[12]

Thus far, microorganisms with theability to producebio-hydrogen have beenisolated.Islama[13] reported ahydrogen yield of 9.11 mmol H_2 g^{-1} a-cellulose by *Clostridium thermocellum* DSM 1237 from 50 g L^{-1} a-cellulose at 60°C.Kadar[14] found that *Caldicellulosiruptorsaccharolyticus* could effectively convert glucose into hydrogen;according to the study, the maximum hydrogen production was dependent on lactate formation.Cao[15] also reported a new strain, namely,*T. thermosaccharolyticum* W16, which could convert xylose and glucose into hydrogen under thermo condition;however,the strain could only degrade

[a] State Key Lab of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China
[b] CAS Key Laboratory of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China

*Corresponding author. Fax: +86 451 86282195.
E-mail address: waj0578@hit.edu.cn (Aijie Wang).

oligosaccharide,whereas the cellulose could not be degraded. Zhang[12] isolated strain *C.sartagoforme* FZ11 froma reactor acclimated by MCC and corn stalk andreported thatthe strain could degradation cellulose under mesophilic culture. According to existing literature, most hydrogen-producing microorganismsprefer oligosaccharide to cellulose; therefore, the development of microbes that can produce cellulosic hydrogen without lignocelluloses hydrolysiscould only be competitive on an industrial scale.

Consolidated bioprocessing (CBP), which combinescellulase production, cellulose degradation, and hexose (or pentose) fermentation in a single bioreactor, is one of the proposed schemesforlignocellulosic hydrogen production. Thermophilic anaerobic bacteria are important in the CBP scheme because of their several advantages, including high efficiency of cellulose degradation,[16] minimal microbial contamination,[17] and wide range of saccharides utilization.[18]

In this paper, we report the isolation and characterization of a novel isolated bacteria strain, *Thermoanaerobacteriumthermosaccharolyticum* DD32, which is capable of rapidly and efficiently producing hydrogen from lignocellulose under thermophilic conditions. Our results suggest that *T.thermosaccharolyticum* str. DD32, which exhibits highly efficient hydrogen production from lignocelluloses by the CBP scheme, could be used as a method for converting lignocellulose into biofuel.

Experimental

Microorganisms and Medium

Deer dung was obtained from the, mudanjiang zoo, China for enrichment culturing as previously reported.[19] Briefly, the dung was mixed then take 1g add 100mL of sterile water purged with N_2. Take the deer dung filtrate through four layers of gauze and stored in a vial purged with N_2 gas prior to use. The filtered deer dung (10% [vol/vol]) was added as an inoculum to 50 mL Mf medium (modified from ATCC 1191 medium replace glucose by microcrystalline cellulose as a substrate (50M; Huka Biochemika 11365; Sigma-Aldrich Chemie) and incubated static at 60°C in a100-mL top-sealed bottle flask. After a 5-day incubation, the inoculated culture broth was added to fresh Mf medium (10% vol/vol) and cultured for another 5 days. When the enrichment process was repeated eight times,tenfoldserial dilutions were placed on the solid MA medium(2%, w/v, agar) prepared in a tube and incubated at 60°Cfor seven days. The agar samples containing well-formedcellulose-clearing colonies were transferred to fresh MAliquid medium under N_2 gas flow and mashed the colonies with sterilized painting stick to liberate cells from the agar.Repeated plating was done multiple times to ensure thepurity of the isolated colonies. Further verification of puritywasensured by microscopy, colony morphology, and16S rRNA gene sequencing. The capability to utilize0.5% v/v cellulose by isolates was observed in batch tests.The Isolate with highest H_2 production potential from cellulosewas used as seed to Mp medium to produce hydrogen. The Mp medium containing 5.0 g L^{-1} Avicel PH-101, 3.0 g L^{-1} KH_2PO_4, 1.5 g L^{-1} $K_2HPO_4 \bullet 12H_2O$, 0.5 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $MgSO_4 \bullet 6H_2O$, 2.0 g L^{-1} yeast extract (YE), 0.5 g L^{-1} L-cysteine, and 1 mL of resazurin (0.2%). The enrichment and cultivation were performed in a Vinyl Type An anaerobic chamber (Coy Laboratory Products, Inc.) containing an 80% N_2-20% CO_2 atmosphere and cultured at 60°C.

Strain identification

Genomic DNA was extracted using a Bacterial DNA Mini Kit (TianGen Biotechnologies Co. Ltd., Beijing, China) according to the manufacturer's instructions. The extracted DNA was used as the template for PCR amplification of the 16S rRNA gene. The 16S rRNA gene was amplified with a pair of universal primers: BSF8/27 (5'-AGAGTTTGATCCTGGCTCAG-3') and BSR1541/1452 (5'-AAGGAGGTGATCCAGCC GCA-3'). (Bioshi, Co., Ltd., Harbin, China). The reaction mixture (50μL) contained 10×PCR buffer(5μl), 0.2 mmol/liter deoxynucleoside triphosphate (dNTP)(2.5μL), 2.5U of Taq DNA polymerase(1.5μL), 0.5molL^{-1}forward primer(2μl) and 0.5 mol L^{-1} reverse primer(2μl), and 20 ng template DNA, The rest filled with double distilled water to 50μL. The samples were amplified using a 9700PCR meter (Bio-Rad Laboratories, Hercules, CA) with the following thermal profile: 95°C for 5min and 35 cycles of 60 s at 94°C, 30s at 60°C, and 1 min at 72°C. The PCR-amplified 16S rRNA was purified, and its size was verified by low-melting-point agarose electrophoresis. Sequencing was performed at the (Sangon Biotechnologies Co. Ltd., Shanghai, China)(http://www.sangon.com.cn). The nucleotide sequences were compared with the sequences in the GenBank/EMBL/DDBJ nucleotide sequence databases by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the Sequence Match program at the Ribosomal Database Project (RDP). Alignment was carried out using ClustalX. Phylogenetic dendrograms were reconstructed using the MEGA program (version 5.1)[20] with the neighbor-joining (NJ) algorithm and bootstrap analysisof 1,000 replicates.[21]

Optimization of culture conditions

To optimize the conditions for degradation cellulose to product hydrogen, the isolated DD32 was tested in batch culture in Mp liquid medium for the following properties: optimal pH, temperature, and the effects of substrate and yeast extract concentrations. The original pH of the Mp liquid medium was adjusted by sterile1M HCl or 1 M NaOH, from 6.0 to 8.5 at intervals of 0.5 before inoculation; the tested temperature was changed from 50°C to 70°C at 5°C intervals. Yeast extract concentrations ranged from 0 to 3.0g L^{-1} at 0.5g intervals. The Avicel was added at five concentrations: 3.0, 4.0, 5.0, 6.0, 7.0 g L^{-1} at 1g intervals. Samples were taken 5mL every 4h to determine the gas production, cell biomass, pH change, reducing sugar and liquid end products.

Hydrogen production from raw lignocellulosic materials under optimal culture conditions

Five different polymeric non-pretreated substrates, buckwheat bran, rice straw, corn stalks, corncob, poplar

branches were chosen as lignocellulosic substrates to test their hydrogen production by *T. thermosaccharolyticum* str. DD32. Lignocellulose biomass were chipped with a Szegvari Attritor System type: B (Union Process Inc.) and screened to 5 mm-38 mm in diameter. Hydrogen production test were performed with 5 g L^{-1} non-pretreated lignocellulosic materials using Mp medium as described in the cultivation tests. 100 mL medium were mixed with 10 mL inoculums (2.8mg dry cells), and kept at 55 ^{o}C for 72 hours. Samples were taken every 4 hrs to determine gas production, cell biomass, pH change, cellulose degraded and liquid end products.

Carbon mass balance.

Carbon mass balance were calculated as output carbon mass divided by input carbon mass:[10] Closure (%) = $([\sum C_{out}]/[\sum C_{in}])\times 100$, where C_{out} is carbon recovery in grams and C_{in} is initial carbon in grams. The evaluation of the carbon mass balance of cellulosic substrates requires information on initial and final carbon contributions, including cellulose concentrations, cell mass concentrations, and soluble protein concentrations, concentrations of sugar, total CO_2 and organic acids. The whole parameters were measured immediately when the inoculation was ending and thereafter every 4 h until the end of the cultivation. The carbon contribution from the medium components (primarily yeast extract) was measured with a CHN analyzer (CHNS/O elemental analyzer 2400; Perkin-Elmer, Norwalk, CT). The carbon content of the soluble proteins was estimated to be 50% (wt/wt) of the total protein mass.[22] The concentration of CO_2 was measured according to the method of Wang[10].

Cellulase activity of DD32

The total cellulase activity was based on the Avicelase determination method described by Wood and Bhat.[23] In brief, the reaction mixture for the enzyme analysis contained 0.5 mL of enzyme solution or culture supernatant and 1.5 mL of 1.0% corresponding substrate in 0.05 mol L^{-1} citrate acid buffer, pH 5.0: carboxymethyl cellulose (CMC) for endo-glucanase activity, avicel cellulose for exo-glucanase activity, salicin for β-1,4-glucosidase activity. After incubation at 60°C for 60 min, the amount of reducing sugars was determined from the absorbance measurements at 540 nm. One unit of enzyme activity (IU) was defined as the amount of enzyme which produced 1 μmol of reducing sugar per 1 min. All assays were performed in triplicate and the mean was reported with standard deviation.

Analytical methods

Cell biomass was estimated using the Bradford method.[24] Cellulose degradation rate was determined according to the method of Huang et al.[25] The voltaic fermentation acids determined by HPLC(4800, Agilent Technologies, USA) as described by Wang et al.[10] Biogas composition was determined by gas chromatography (102G, Shanghai Analysis conductivity detector (TCD). H_2 was detected by a stainless steel column packed with Molecular Sieve 5A. Nitrogen was used as carrier gas at a rate of 25 mL min^{-1}. The temperatures in column, injection, and detector were 110^{o}C, 205^{o}Cand 300^{o}C, respectively.

Results and discussion

Isolation and Identification of a cellulosic-hydrogen production strain DD32

An enrichment culture was established by incubating deer dung in an Avicel mineral salt medium with 2.0g L^{-1} yeast extract (YE) at pH 7.0 and 60°C. One bacterial strain called DD32 was isolated.This strainwas able to directlyproduce hydrogen in the cellulose. An analysis of the 16S rRNA gene sequence of this strain indicates that the strain is a member of genus *Thermoanaerobacterium*. A phylogenetic analysis of 16S rRNA genes shows that DD32 has a 99% sequence identity to the 16S rRNA gene of *Thermoanaerobacteriumthermosaccharolyticum* M2 (Figure 1). The physiological properties of *T.thermosaccharolyticum* str. DD32 are summarized in Table 1. Strain DD32 was able to utilize cellulose to produce H_2 and to hydrolyze various cellulosic materials, such as Avicel, CMC, and filter paper. Some species of *thermosaccharolyticum* spp., including *T. thermosaccharolyticum* GD17and*T. thermosaccharolyticum* W16, are able to ferment glucose, fructose, and so on,except for cellulosic materials. Similar toother strains of the genus *thermosaccharolyticum*, DD32 cells are oval shaped (Figure S1), tufted flagellum (0.5–0.7μm by 1.0–1.4μm), motile, and exhibit anaerobic growth but do not reduce sulfate or nitrate (Table 1).

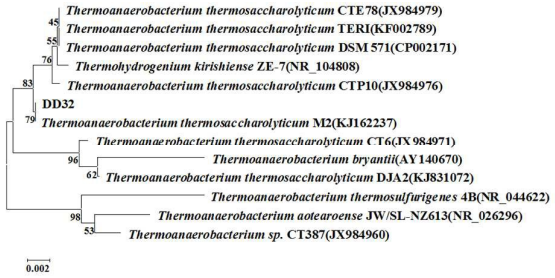

Figure 1 Phylogenetic relationship of strain DD32 and other known Thermoanaerobacterium strains based on 16S rRNA gene sequences. Numbers along branches indicate bootstrap values with 1000 times.

Table 1 Physiological properties of T. thermosaccharolyticum DD32

Characteristic	Value†	Substrate utilization	Value†
Gram staining	+	Avicel	+
		Glucose	+
		Fructose	+
		Xylose	+
		Maltose	+
		Lactose	-
Morphology	Short rod-shaped, spore	Sucrose	+
Anaerobic growth	+	Cellobiose	+
Motility	+	Xylitol	+
Sulfate reduction	-	CMC	+
Nitrate reduction	-		
Gelatin hydrolysis	+	Starch	+
Metabolic products with cellulose	Acetate, Butyrate, Ethanol, Butanol Hydrogen	Filter paper	+

†,-negative; +positive.

Key factors affecting cellulosic-hydrogen by strain DD32

Temperature and pH

Fermentative hydrogen production is not always the same.In the current research, production fell into the mesophilic range (at approximately 37°C) and thermophilic range (at approximately 55 °C).[26]Therefore, temperature is a key factor for cellulose degradation by a thermophilic anaerobic microorganism. As shown in Figure 2, both hydrogen production and cellulose degradation rates increased with increasing temperature in the range of 50°C to 55°C and then decreased when the temperature increased from 55°C to 70°C. No cellulose degradation was observed at temperatures below 50°C or above 65°C. The optimal temperature for hydrogen production was 55°C. Moreover, both the maximum rates of cellulose degradation and cell concentration were obtained at 55°C. Thisresult was similar to that of*T. thermosaccharolyticum* sp.[27, 28] The maximum hydrogen production, cell concentration, and cellulose degradation obtained at 55°C were 12.78±0.02mmol H_2 g^{-1}, 0.27±0.02g L^{-1}, and81.25±1.03% respectively. The finding suggests that 55°C is not only the optimum bio-hydrogen production condition but also the optimum condition for DD32 growth. On the basis of the above results, 55°C is selected as the optimum temperature in the following batch cultures.

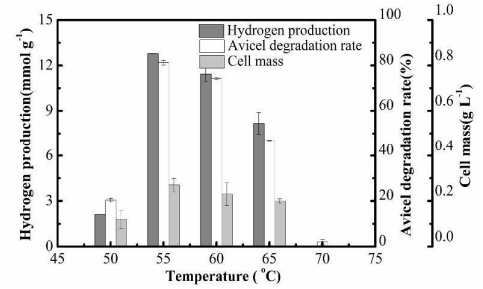

Figure 2. Effect of temperature on hydrogen production by T. thermosaccharolyticum

pH plays an important role in determining the type of anaerobic fermentation pathway in anaerobic bio-hydrogen processes,[29] and appropriate pH will facilitate the hydrogen production.[30] Figure3 presentsthe effect of theinitial pH value of medium on hydrogen production in the range of 6.5–8.5 at a fixed Avicel of 5 g L^{-1}. As presented in Figure 3, hydrogen production and cell degradation increased with increasinginitial pH value ranging from 3.16±0.28mmolg^{-1} and 37.13%±1.08% at aninitial pH of6.5 to the maximum value of12.14±1.39 mmolg^{-1} and 82.04%±2.19% at an initial pH of 7.5, and then gradually decreased with increasinginitial pH value ranging from 9.78±0.94 mmolg^{-1} and 71.88±3.75% at pH 8.0 to 5.22±0.61 mmolg^{-1} and 39.44±2.99% at pH 8.5. The similar changing trend was also observed for cell protein.The highest cell protein concentration occurred at initial pH 7.5. As soon as the pH value deviated from the optimal level of 7.5, the cell protein concentration decreased. Our finding is similar tothat ofMielenz and Ahmad,[31, 32] in which the optimal initial pH for hydrogen production usually reaches a neutral level. The results indicated that the reasonable control of medium pH was significant for improving hydrogen production because the activity of hydrogenase could be inhibited, and the corresponding metabolic pathway would be changed by low or high pH values in overall hydrogen fermentation process.[12]

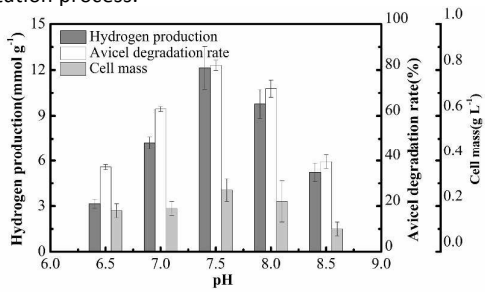

Figure 3. Effect of pH on hydrogen production by T. thermosaccharolyticumDD32

Yeast extract concentration

Many studies applied rich nitrogen media (i.e.YE) for the lignocellulose hydrogen production process in laboratories because lignocellulosic substrates possess high C/N ratios. However, the excessive manual addition of YE will increase the cost of hydrogen production from cellulose.[10]Furthermore, hydrogen production would be inhibited by high nitrogen source concentration.[12] Therefore, we investigated the optimal YE

concentration for DD32 product hydrogen by cellulose. DD32 was grown in a batch culture at 5.0 g L^{-1} of Avicel, 55°C, and pH 7.5 with varied concentrations of YE. As shown in Figure 4, hydrogen production was improved when the YE concentration increased from 0 g $L^{-1}$to 3 g L^{-1}. A similar profile was observed for the cell mass. However, hydrogen production rate was getting much slower when the YE concentration increased over 1.5 g L^{-1}. Furthermore, the cellulose degradation rate gradually decreased with the addition of YE when the YE concentration was beyond 1.5gL^{-1}. The maximum Avicel degradation ratio was obtained when theYE concentration was 1.5 g L^{-1}; the hydrogen production and cellulose degradation rate were 12.06±2.19 mmol H_2 g^{-1}Avicel and 82.39%±3.19%, respectively.

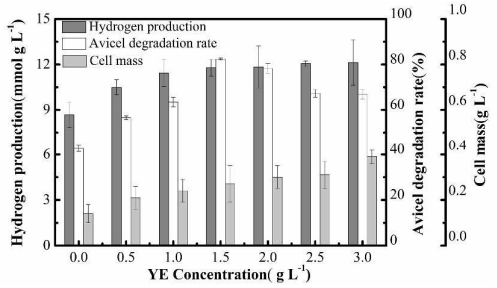

Figure 4. Effect of Yeast concentrations on hydrogen production by T. thermosaccharolyticum

Avicel concentration.

The maximum hydrogen production was closely related to cellulose degradation activity[33].The microorganism degraded the cellulose by synthesizing cellulosome, which was saturable to the substrate. Excessive substrate will binding the active site of cellulase and result a poor hydrolysis performance overall. The process of DD32 yield hydrogen includes cellulose hydrolysis and hydrogen production two steps, and the inefficiency cellulose hydrolysis limited the hydrogen production.Therefore,the Avicel degradation capability under different substrate concentrations was determined. *T. thermosaccharolyticum* DD32 was grown on Avicel with substrate concentrations ranging from 3.0 g $L^{-1}$to 7.0 g L^{-1}(Figure 5). Approximately 91% Avicel degradation was achieved at 3.0 g L^{-1}, but this percentage decreased to 52% at 7.0 g L^{-1}. Unlike the change in cellulose degradation rate, cell protein remained constant whenAvicel concentrations changed from 3.0 g L^{-1} to 7.0 g L^{-1}.Cell protein wasalso constant at approximately 280 mg L^{-1}. Both the maximum Avicel degradation ratio and hydrogen yield were reached.

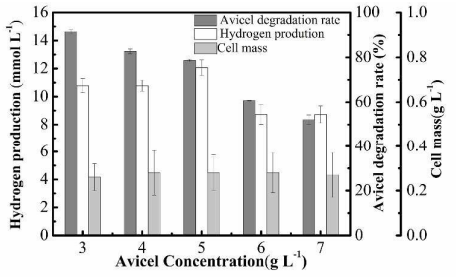

Figure 5. Effect of Avicel concentrations on hydrogen production by *T. thermosaccharolyticum*

Characterization of the cellulose degradation and hydrogen production under optimum culture conditions.

As shown in Figure 6, when the inoculum of strain DD32 was cultivated in the 5g L^{-1}Avicel mineral salt medium at 55°C andpH 7.5, hydrogen production began after a4h lag phase.The H_2production rate kept increasingfor 20h and reached the maximum hydrogen production rate of approximately 3.0 mmol $h^{-1}L^{-1}$ (date not shown).Thereafter, the production rate rapidly decreased. By contrast, hydrogen accumulation gradually increased. A maximum hydrogen production of 12.04±0.21mmol H_2 g^{-1}Avicel was obtainedat approximately48handremained steady thereafter. The cellulose concentration profilewas consistent with hydrogen production, and a trace of cellulose degradation was obtained before its formation;this degradation could be the cause of cellulose hydrolysis in hot water.[34]The cellulose concentration gradually decreased with hydrogen accumulation, which ranged from 4.91±0.03 g L^{-1} at 0h to 1.44±0.03 g L^{-1} at 48h.Cellulose degradation almost remained static48h later because of either the depletion of a particular nutrient from the culture medium[22] or the accumulation of inhibitory intracellular compounds in the medium/cell.[27, 28]Different from the cellulose degradation rate, the cell protein concentration gradually increased before 24h and then remained steady. The cell protein concentration reached the peak earlier than hydrogen production and cellulose degradation, thus suggestingthat microorganism biologicalactivityand not microorganism growth was the key factor in hydrogen production.

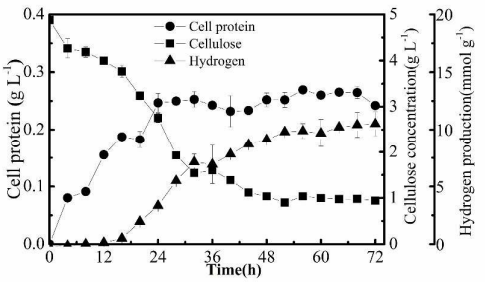

Figure 6. Kinetics of Hydrogen yield, cellulose degradation and cell protein over time at 5.0 g L-l Avicel, 1.5 g L-lYeast, pH7.5, 55oC within 72h of batch fermentation.

HPLC analysis showed that theprimary products of fermentation included acetate, butyrate, ethanol,butanol, and trace amounts of isobutyrate and propionate, whereas no lactate was determined. The metabolites appearedduring hydrogen production. As shown in Figure7, the metabolites were produced during hydrogen production and gradually increased until the 48th h; the main type of metabolites was acetate, flowed by butyrate, ethanol, and butanol. The ratio of acetate to butyrate was 2/1, thus suggesting that the bio-hydrogen of DD32 wasproduced bybutyric-type fermentation.[35]

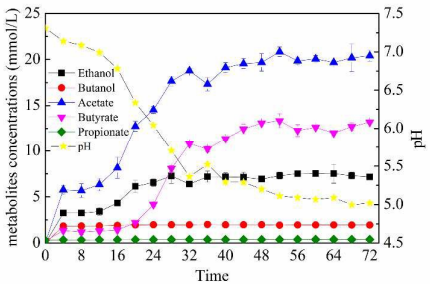

Figure 7. Metabolites of DD32 degradation cellulose over time at 5.0 g L- l Avicel, 1.5 g L- l Yeast, pH7.5, 55oC within 72h of batch fermentation.

The cellulase activity of DD32 under optimum culture conditions is shown in Figure8. The cellulose activitieson both the surface and external structureof the cell were obtained. In general, the cellulase activity on the cell surface was higher than that of the extracellular one. The cellulase activity on the cell surface was obtained after 4 h; the activity gradually increased, reached the peak at 24h (Figure 8a), andthen gradually decreased; this resultwas consistent with the hydrogen production rate and cell protein trends.The maximum cellulase activity obtained at 24h was exo-1,4-β-D-glucannase(0.67U), followed by endo-1,4-β-D-glucanas(0.37U), and β-1,4-glucosidase(0.32U). The extracellular cellulase activity reached the maximum value at 32h(Figure 8b), which was different from cell cellulase activity.As reported in literature, cellulose degradationby bacteria was achievedvia cellulosomesynthesison the cell surface and the extracellular cellulose by shedding cellosomefrom the cell surface[36, 37]; therefore, the extracellular cellulose occurred first, followed by the cell cellulase. The extracellular maximum cellulase activity was exo-1,4-β-D-glucannase(0.41U), followed by β-1,4-glucosidase(0.34U) and endo-1,4-β-D-glucanas(0.33U).

All aforementioned results indicated that *T. thermosaccharolyticum* DD32 exhibits high cellulose hydrolysis activity and hydrogen production yield (Table 2).

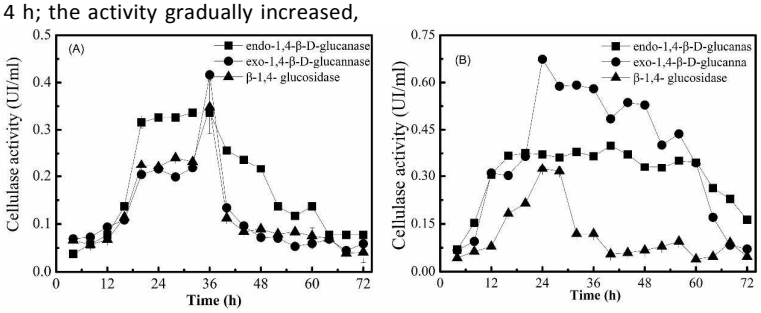

Figure 8. The cellulase activity of DD32 during 72h of batch fermentation at 5.0 g L- l Avicel, 1.5 g L- l Yeast , pH7.5, 55oC. (a) Extracellular cellulase activity. (b) Cell cellulase activity.

Table 2. Comparison of hydrogen production from various cultures with cellulose as substrates

Microorganism	Substrate	Concentration ($g\ L^{-1}$)	Temperature (°C)	Hydrogen Production ($mmol\ g^{-1}$)	References
***C.thermocellum* JN4 +*T. thermosaccharolyticum* GD17**	Microcrystalline cellulose	5.0	60	10.00	27
***C.saccharolyticus* DSM 8903**	Microcrystalline cellulose	2.0	65	9.40	38
***C. thermocellum* DSM 1237**	a-cellulose	10.0	60	10.12	22
***T. thermosaccharolyticum* M18**	Microcrystalline cellulose	5.0	60	10.90	19
***T. thermosaccharolyticum* DD32**	Microcrystalline cellulose	5.0	55	12.06	This study
C.saccharolyticus	Cellulose	4.5	65	11.33	39
***Thermoanaerobacter* GHL15**	Cellulose	4.5	65	7.22	39
***Clostridium thermocellum*27405**	Cellobiose	4.5	60	3.78	40

Carbon balance.

Carbon balance of *T. thermosaccharolyticum* DD32 was further evaluated based on the utilization of Avicel and the production of cell biomass (total protein), oligosaccharides, liquid end products, and CO_2. The carbon closures ranged from 94.59% to 99.70% for it (Figure S2). Initially, C balance closures

of (99.70%±0.3%) were observed at 8 h, C balance closures decline slightly to 97.3%±0.21% at 40h, and 94.55±0.34 at 55h. Carbon balance analysis indicated that volatile acid and cell are the dominant components in the later stages. About 54% of the total carbon was converted to acetate, butyrate, and the remainder was used for the production of CO_2, ethanol and cell mass. This carbon partition was quite similar from that in the other studies. Analysis of carbon flow in *C. cellulolyticum* showed that 55% to 70% of the degraded cellulose was converted into end products, such as acetate and lactate, and the rest was converted into biomass, polysaccharides, and proteins [41]. In contrast, the carbon balance of a cellulosic saccharificaiton strain *Shigella flexneri* G3 showed that 45% of total carbon was converted to oligosaccharides and the remainder was for the production of volatile fatty acid (VFA), CO_2, and soluble protein,[10] while the production of hydrogen was near zero. It is believed that the type of organic acid decided the end products, for example, acetate butyrate often companies with hydrogen but lactate not, that's because no H^+ reduced into hydrogen during cellulose hydrolyzed into lactate. It is obviously that the carbon was flow into acetate and butyrate mainly, and no carbon transfer to lactate, that's also explained the DD32 was effective on hydrogen production.[42-45]

In short, the analysis of carbon balances indicated that DD32 capability of efficient hydrogen production capacity than other known cellulosic microorganisms.

Hydrogen production from natural (raw) lignocellulosic feedstock by strain DD32

To assess the ability of T.thermosaccharolyticum DD32 in converting lignocelluloses into hydrogen, non-pretreatedlignocellulosic biomass utilizationwas investigated in strain DD32. Five raw lignocellulosic materials, namely, buckwheat bran, rice straw, corn stalks, corncob, and poplar branches, were used as substrates to test their hydrogen production. In general, considerable high yields of H2 were generated from all substrates. Strain DD32 was able to utilize carbohydrates in raw lignocellulosic materials as asource for H2 production. The hydrogen yield from various raw lignocelluloseshas the following order: corn stalks > corncob > rice straw > poplar branch> buckwheat bran (Figure 9). The highest hydrogen production of strain DD32 was 6.38±0.41mmol H2 g−1from corn stalks. In comparison, the H2 yield of C. saccharolyticus DSM 8903 was 1.58 mmol H2 g−1from wheat straw,32 the H2 yield of C.thermocellum 7072 was 2.76 mmol H2g−1from corn stalk,12 and the H2 yield from corn stalk by T. thermosaccharolyticum M18 was 3.28 mmol H2 g−1, which was the highest.19 The above results suggested that the hydrogen yield of T. thermosaccharolyticum DD32 from lignocelluloses was 2–3 times higher thanthe other strains (Table 3).

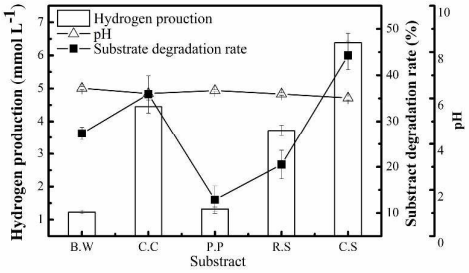

Figure 9. Hydrogen production with lignocelluloses feedstock at 5.0 g L-1subtract, 1.5 g L-1 Yesat, pH7.5, 55oC within 72 h of batch fermentation of T.thermosaccharolyticum DD32.

B.W: buckwheat bran; C.C: corncob; P.P: poplar branche; R.S: rice straw; C.S: corn stalks

Table 3.Comparison of hydrogen production from various cultures with lignocellulose as substrates

Microorganism	Substrate	Concentration (g L-1)	Temperature (oC)	Hydrogen Production (mmol g-1)	References
***C.thermocellum* 27405**	delignified wood	4.5	60	3.55	46
***C. saccharolyticus* DSM 8903**	Wheat straw	10.0	70	1.58	47
***C. saccharolyticus* DSM 8903**	Switchgrass-unwashed	30.0	65	0.47	38
***T.thermosaccharolyticum* M18**	Corn cob	5.0	60	3.23	19
***T. thermosaccharolyticum* DD32**	corn straw	5.0	55	6.38	This study
***C.thermocellum* 7072**	Corn stalk	20.0	55	2.76	40
***Thermoanaerobacter* GHL15**	Hemp stem (hydrolysates)	4.5	65	5.62	39
***Thermoanaerobacter* GHL15**	Grass (hydrolysates)	4.5	65	6.22	39
***C.thermocellum* 27405**	dried distillers grain	5.0	60	1.07	48

Conclusion

In this study, a novel thermophilic bacterium *T. thermosaccharolyticum* DD32 was isolated from deer dung and thencharacterized. DD32 was capable of efficiently degrading

cellulose and producing hydrogen. To the best of our knowledge, thermophilic cellulolytic bacterium has the highest capability for producing hydrogen capability from cellulose. This strain could be potentially useful for various types of biotechnologies in hydrogen production, waste treatment, and energy production from cellulosic materials.

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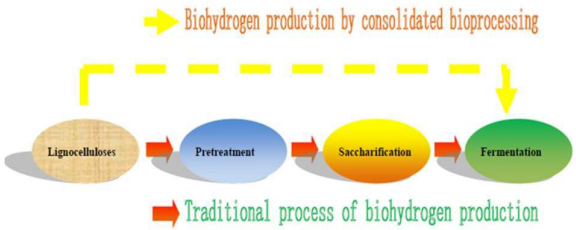

The comparison of hydrogen production by conventional process and consolidated bioprocessing