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Fermentative hydrogen production from corn stover hydrolyzate by two typical seed sludges: Effect of temperature

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ABSTRACT

The temperature effect on fermentative hydrogen (H_2) production from corn stover hydrolyzate was investigated under mesophilic (37 and 30 °C), thermophilic (55 °C), and extreme thermophilic (70 °C) conditions by using two typical seed sludges (activated sludge and anaerobic granular sludge). Among various temperatures, the fermentation at 55 °C reached the optimal H_2 production with the values of 6.08 mmol- H_2 /g-utilized sugar for activated sludge and 7.74 mmol- H_2 /g-utilized sugar for anaerobic granular sludge, respectively. For the two seed sludges, the effectiveness of fermentation temperature on H_2 production both followed the order as 55 °C > 70 °C > 37 °C ≈ 30 °C. The soluble metabolites composition at 55 °C showed the highest acetate and butyrate concentrations, as well as the minimum ethanol production, coinciding with better H_2 -producing performances in these cases. Microbial community analysis indicated that microbial community diversity significantly decreased with increased fermentation temperature. Facultative anaerobes, such as *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp., were dominant in microbial community of the two seed sludges. As efficient H_2 producers, *Bacillus* sp. AB5283 in activated sludge and *Thermoanaerobacterium* sp. PO-2009 in anaerobic granular sludge might be mainly responsible for high H_2 yields under thermophilic condition.

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Introduction

Due to the depletion of fossil fuels and the aggravation of environmental pollutions, the development of clean and renewable energy sources has become an important part of fundamental energy strategy in many countries [1,2]. Among various novel energy vectors, hydrogen (H_2) attracts much

attention due to its high energy content (142 kJ/g), non-polluting nature (carbon dioxide-neutral and no gaseous pollutants emission), and versatility in many fields (direct combustion, gas engine, electricity production by fuel cells, etc.) [3]. Traditionally, H_2 is industrially produced by energy intensive processes, such as steam reforming of natural gas, gasification of coal, and water electrolysis [4]. In recent years, dark fermentative H_2 production has become a promising

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method for sustainable practical applications owing to simple reactor configuration, mild operation conditions, and high H_2 production rate [5]. More importantly, H_2 production by dark fermentation can be achieved by using various organic waste materials in an eco-friendly way, by which H_2 production cost can be significantly decreased to make it economically feasible at a commercial scale [6].

To explore suitable substrates for H_2 production, many kinds of organic wastes have been examined for their H_2 -producing potentials, such as sugar-rich feedstock [7], kitchen wastes [8], biodiesel and oil residuals [9], and lignocellulosic material [10]. Among these wastes, lignocellulosic material is one of the most promising substrates by virtue of the fact that they are abundant, easily available, and low-cost [11,12]. In order to decompose the harsh microstructure of lignocellulosic feedstock to make it more microbially accessible, lignocellulosic hydrolyzate is commonly generated and recognized as an applicable utilization form. However, due to its complex composition with various monosaccharides and byproducts, H_2 yields are found to be relatively low [13]. For the purpose of improving H_2 yield by using lignocellulosic hydrolyzate, some researchers pay more attention to optimizing fermentation conditions for mixed anaerobic microflora, such as nutrient, temperature, and pH [14–16]. By doing this, effective H_2 -producing microbes capable of decomposing complex organic compounds are expected to be predominant in microbial community, leading to better performances on H_2 production and substrate utilization efficiency.

Among various parameters, temperature is a vital factor for dark fermentative H_2 production, affecting growth rates and metabolic activities of H_2 -producing microbes [17]. Generally, fermentative H_2 production can be maintained at mesophilic (25–40 °C), thermophilic (40–65 °C), and extreme thermophilic (65–80 °C) conditions [18]. For a long time, mesophilic conditions have been commonly adopted for fermentative H_2 production. Recently, thermophilic and extreme thermophilic conditions attract much attention for H_2 production because of several advantages, such as efficient utilization of complex substrates, better thermodynamic conditions, and suppression of methanogens [19]. Moreover, the predominance of some efficient H_2 -producing thermophiles, such as *Thermoanaerobacterium* spp., is considered as key microbial factor responsible for better performances in these cases [20]. Till now, some studies have been conducted to comparing temperature effects on H_2 production by using various wastes, such as palm oil mill effluent [21], *Laminaria japonica* [22], and feedlot cattle manure [23], but few studies are reported to explore preferable temperature with lignocellulosic materials as the substrate [24,25]. In addition, due to different fermentation patterns and bacterial community structures in these studies, we can hardly make clear the inherent relationships among fermentation temperature, H_2 -producing performance, and microbial community characteristics.

In this study, two commonly-used seed sludges, i.e., activated sludge from a municipal treatment plant and anaerobic granular sludge from an anaerobic bioreactor, were used as touchstones to investigate the effect of temperature on dark fermentative H_2 production and microbial community structure. With corn stover hydrolyzate as the substrate,

fermentation tests were kept at mesophilic (37 and 30 °C), thermophilic (55 °C), and extreme thermophilic (70 °C) conditions, respectively. Biogas and H_2 production were measured to address H_2 -producing behavior, by which kinetic analysis was carried out using the modified Gompertz equation. Soluble metabolites were investigated to reveal relationships between H_2 production and distribution of liquid end products. Microbial community structure was analyzed by using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and corresponding biodiversity analysis.

Materials and methods

Raw materials and the hydrolysis process

Corn stover feedstock was obtained from the suburb of Harbin city, Heilongjiang Province, China. The raw corn stover was thoroughly washed with clean tap water to remove impurities and naturally desiccated at room temperature. Subsequently, dried corn stover was grinded to a particle size of 0.2–0.4 mm by using a tissue crusher (SZ-1100B-3, Shangzu, China). The main composition of the corn stover powder contained 38.7% glucan, 20.3% xylan, 18.2% lignin and 4.2% ash. For the following acid hydrolysis process, the corn stover powder was put into 1.7% (v/v) H_2SO_4 solution with a solid/liquid ratio of 1:50 (w/v). The hydrolysis time and temperature were kept at 120 min and 121 °C, respectively. After the hydrolysis process, solid residues of the suspension were removed by centrifugation at 12,000 r/min for 5 min. By adjusting the supernatant to pH 7.0 with 1 mol/L NaOH solution, corn stover hydrolyzate was generated with the reducing sugar concentration of about 6.2 g/L. The raw corn stover hydrolyzate was then prepared for the following anaerobic fermentation.

Seed sludge and batch test

Two typical seed sludges were used in this study, namely, activated sludge from an aeration tank of Wenchang wastewater treatment plant, and anaerobic granular sludge from a bench-scale expanded granular sludge bed reactor (EGSB) treating starch wastewater. For revealing the effect of temperature on original microbial community truthfully, commonly-used pretreatment methods (such as boiling) were not carried out on the two seed sludges. After the removal of impurities by using sieves, the pH, total suspended solid (TSS) and volatile suspended solid (VSS) concentrations were 6.86, 5.77 g/L, and 4.21 g/L for activated sludge, and 7.58, 39.3 g/L, and 28.9 g/L for anaerobic granular sludge, respectively. For the following fermentation tests, the inoculation dosage was about 4% (v/v) by using diluted seed sludges, and the microbial concentrations in medium were about 0.16 g-VSS/L for activated sludge inoculation and about 0.96 g-VSS/L for anaerobic granular sludge inoculation, respectively.

Fermentation tests were carried out anaerobically in 100 mL serum vials at a working medium volume of 50 mL. For all the tests, the medium contained 5.0 g/L sugars diluted from corn stover hydrolyzate (about 3.8 g/L xylose, 0.9 g/L glucose, 0.2 g/L arabinose, and 0.1 g/L other sugars), and supplemented

The experimental results indicate that with complex corn stover hydrolyzate as the substrate, fermentation at 55 °C led to the optimum H₂ yield for the two seed sludges, which was in accordance with previous studies. Kumar et al. [33] collected the seed sludge from a municipal wastewater

Fig. 1 – Time-course profiles of the biogas and H_2 production under different fermentation temperatures. (A) activated sludge; (B) anaerobic granular sludge.

treatment. By using cellulosic materials-based de-oiled *Jatropha* waste, they concluded that the peak H_2 production rate and H_2 yield were both obtained at 55 °C. Cakır et al. [24] obtained the seed sludge from acidogenic phase of an anaerobic wastewater treatment plant. With the inoculum pretreatment (boiling at 100 °C for 1 h) and the acclimation process (55 °C for 3 d) for the seed sludge, they found that dark fermentation of acid-hydrolyzed ground wheat was more beneficial under thermophilic condition (55 °C) than mesophilic condition (37 °C). Actually, by the inoculation of mesophilically and thermophilically digested sludge, thermophilic acidogenic sludge, cow waste slurry, etc., peak H_2 yields can be commonly achieved under thermophilic environments (55–60 °C) with other complex wastes as the substrate [34,35]. In our view, better H_2 -producing performances under thermophilic conditions can be attributed to selective enrichment of some efficient H_2 -producing thermophiles, which are capable of producing more H_2 by utilizing complex substrate components (to be illustrated in section 3.4).

In this study, extreme thermophilic condition (70 °C) led to relatively lower H_2 production in comparison with thermophilic condition (55 °C). In some other studies, Kongjan et al. took anaerobic sludge from a continuous stirred tank reactor (working temperature of 70 °C) as the inoculum, and they made repeated batch cultivations for the acclimation of the seed sludge [36]. Liu et al. obtained the seed sludge from H_2 fermentation reactors using glucose and synthetic BA media under 70 °C [37]. In these studies, high H_2 yields were achieved under extreme thermophilic environment (70 °C) with pentose and other wastes as the substrate, and the authors attributed the better H_2 -producing performance to preferable thermodynamic conditions, rapid substrate hydrolysis rate, and the enrichment of some special extreme thermophiles [38]. In our eyes, the unsatisfactory H_2 -producing performance under extreme thermophilic environment in this study might be mainly due to the fact that efficient H_2 -producing microbial community was not successfully founded in this case. Indigenous extreme thermophiles in the two seed sludges might be weak in utilizing complex substrate components, and negatively affected by some byproducts within corn stover

hydrolyzate. For mesophilic conditions (37 and 30 °C), H_2 yields were significantly low in this study, suggesting that mesophilic conditions might not be suitable for biohydrogen production by using lignocellulosic hydrolyzate.

As widely-used seed sludges for wastewater treatment and biogas production, the chosen activated sludge and anaerobic granular sludge in this study were representative to serve as ideal touchstones to evaluate the effect of fermentation temperature on H_2 production from corn stover hydrolyzate. With 55 °C as the optimal fermentation temperature for the two seed sludges, thermophilic condition corresponded to an enhanced H_2 production from anaerobic granular sludge (3.6 times of that at 37 °C) than activated sludge (2.2 times of that at 37 °C). Under mesophilic conditions (37 and 30 °C), activated sludge inoculation resulted in more H_2 production than anaerobic granular sludge.

Kinetic analysis

Kinetic analysis was carried out for understanding H_2 -producing behavior under different fermentation temperatures. By using the modified Gompertz equation, we calculated important parameters, characterizing the H_2 production processes (Table 1). With the values of R^2 all larger than 0.99, the H_2 accumulation trend of all batch tests could be adequately described by the equation. We found that the kinetic parameters were in accordance with H_2 -producing behaviors described in section 3.1. Concretely, for the two seed sludges, the optimum H_2 production potential and maximum H_2 production rate were both achieved at 55 °C (activated sludge: 627.4 mL/L and 30.3 mL/h; anaerobic granular sludge: 821.8 mL/L and 43.3 mL/h), indicating that the thermophilic conditions favored enhancement of H_2 production while utilizing complex corn stover hydrolyzate. For lag-phase time, fermentation temperature at 30 °C corresponded to the shortest lag-phase time among the various temperatures tested (activated sludge: 10.7 h; anaerobic granular sludge: 11.0 h), suggesting that indigenous microbes can rapidly adapted to the complex components of corn stover hydrolyzate for H_2 production. For much higher fermentation

Table 1 – Kinetic parameters for H₂ production under different fermentation temperatures.

Seed sludge	Temperature (°C)	P (mL/L)	R _m (mL/h)	λ (h)	R ²
Activated sludge	37	275.8 ± 3.3	16.5 ± 0.9	15.3 ± 0.5	0.997
	30	263.4 ± 2.7	17.4 ± 1.0	10.7 ± 0.5	0.996
	55	627.4 ± 10.8	30.3 ± 2.0	12.6 ± 0.7	0.994
	70	506.6 ± 37.1	12.5 ± 0.7	21.3 ± 0.9	0.993
Anaerobic granular sludge	37	223.6 ± 3.3	18.2 ± 1.7	15.4 ± 0.6	0.993
	30	235.4 ± 3.3	16.7 ± 1.4	11.0 ± 0.6	0.993
	55	821.8 ± 13.9	43.3 ± 2.9	16.6 ± 0.6	0.995
	70	463.1 ± 4.2	28.0 ± 1.3	13.8 ± 0.4	0.998

Values represent average ± standard deviation. P: maximum H₂ production; R_m: maximum H₂ production rate; λ: lag time.

temperatures (37 °C, 55 °C, and 70 °C), long lag-phase time was necessary for indigenous microbes to acclimate the fermentation conditions prior to substrate utilization and biohydrogen production.

Soluble metabolites profiles

Fermentative processes with H₂ production are always associated with the generation of VFAs and alcohols, and these soluble metabolites are interrelated with H₂ formation on the basis of NADH balance [39]. At the end of the fermentation, we measured the concentrations of various liquid end products, including ethanol, acetate, propionate, butyrate, and formate (Fig. 2). Soluble metabolites profiles for each batch test performed a wide spectrum of various organic acids and ethanol, which might be the result of decomposing complex components within corn stover hydrolyzate. The hydrolysis process of corn stover produces many kinds of monosaccharide (glucose, xylose, arabinose etc.) and byproducts such as furan derivatives and phenolic compounds [40]. While utilizing these compounds, various metabolic pathways might be induced and result in multiple soluble metabolites [41]. At the same temperature, soluble metabolites profiles were similar for the two seed sludges, which might be attributed to similar microbial composition at the end of the fermentation (to be illustrated in section 3.4), which performed similar utilization patterns towards corn stover hydrolyzate.

For the two seed sludges, the fermentation temperature surely changed the distribution of soluble metabolites profiles. The fermentation process at 55 °C corresponded to the

highest acetate and butyrate concentrations, and the minimum ethanol production, performing typical butyrate-type fermentation. Since more acetate and less ethanol production mean that more NADH can be efficiently utilized for H₂ production, the soluble metabolites profiles at 55 °C was in accordance with better H₂-producing performance in this case. At 37 °C, 30 °C and 70 °C, all the tests performed mixed acid fermentation, meaning that a large amount of NADH was used for liquid products other than H₂, such as propionate [42]. In addition, we observed some formate production at 30 °C, 55 °C, and 70 °C, suggesting that some H₂ might be generated via formate cleavage pathways in these cases [43].

Microbial community structure

In this study, PCR-DGGE and corresponding microbial diversity analysis were carried out to elucidate internal relationships between fermentation temperature and microbial community structure. Fig. 3 graphically displays different DGGE profiles of 16S rDNA fragments for microbial samples under different temperatures. For the fermentation process of corn stover hydrolyzate, microbial community structure was simple for the two seed sludges, especially for anaerobic granular sludge. The phenomenon might be mainly due to the selective enrichment of specific species capable of utilizing refractory sugars in stover hydrolyzate. In addition, it has been convinced that toxic byproducts in corn stover hydrolyzate can cause negative efforts on many species in seed sludges [27,44]. Under the situation, microbial community diversity unavoidably decreased with the elimination of non-

Fig. 2 – Soluble metabolites components under different fermentation temperatures. (A) activated sludge; (B) anaerobic granular sludge.

Fig. 3 – DGGE profiles of 16S rDNA gene fragments from samples under different fermentation temperatures. (A) activated sludge; (B) anaerobic granular sludge.

adaptable species and the enrichment of some functional microbes.

Fermentation temperature caused significant influences on microbial components. For activated sludge (Fig. 3A), fermentation process at 37 °C and 30 °C determined a complex microbial community with most species were present only with small quantities. However, microbial community structure at 55 °C and 70 °C showed significant differences from that at mesophilic conditions, being characterized by selective enrichment of some specific species (band A16 and A22 at 55 °C, band A16 at 70 °C, etc.). For anaerobic granular sludge (Fig. 3B), microbial community diversity was relatively low under various temperatures. We assumed that the result might be attributed to simple microbial community structure in anaerobic granular sludge. In addition, microbial community was almost the same for 37 °C and 30 °C, and was less similar to that at 55 °C and 70 °C. The enrichment of a microbe (band G14) was found among various temperatures, suggesting that the strain might be well adapted to grow at a wide temperature range and act an important role due to its abundance.

Based on the digital recognition of dominant DGGE bands, number of bands, Shannon–Wiener diversity index, Simpson diversity index, and Pielou evenness index were calculated to address microbial community characteristics (Table 2). For the two seed sludges, more DGGE bands can be detected under mesophilic conditions (37 and 30 °C), and number of bands significantly decreased under high temperatures (55 and 70 °C). Shannon–Wiener diversity index and Simpson diversity index both performed a decreasing trend with the increase of fermentation temperature (except 30 °C for activated sludge), indicating that the higher the fermentation temperature, the lower the microbial community diversity. The trend was also reported by previous studies [22,45,46], and the reason might be possible susceptibility of many microbial species towards high temperatures. Pielou evenness index also showed similar variations with the other two diversity indexes. Low Pielou evenness index under high temperatures suggested that few species succeeded in dominating the microbial community. In these cases, high H₂ yields might be in strong association with the behaviors of enriched species. Due to better H₂-producing performances under high

Table 2 – Microbial diversity parameters for H₂ production under different fermentation temperatures.

Seed sludge	Temperature (°C)	Number of bands	Shannon–Wiener diversity index	Simpson diversity index	Pielou evenness index
Activated sludge	37	14	2.334	0.868	0.884
	30	9	1.626	0.706	0.740
	55	9	1.838	0.791	0.836
	70	6	1.122	0.525	0.626
Anaerobic granular sludge	37	11	1.986	0.808	0.828
	30	11	1.920	0.791	0.801
	55	8	1.285	0.586	0.618
	70	5	1.020	0.560	0.633

temperatures, the predominance of these species might be more beneficial for H₂ production.

Prominent DGGE bands were sequenced and then aligned to determine their phylogenetic affiliation (Table 3 and Table 4). In the two tables, most bands (32 of 39) presented more than 97% similarities with known strains, indicating that they might belong to the same species. For the two seed sludges, facultative anaerobes were frequently detected in microbial community (activated sludge: *Enterobacter* spp. and *Klebsiella* spp.; anaerobic granular sludge: *Klebsiella* spp. and *Citrobacter* spp.). These facultative anaerobes might be partly responsible for H₂ production by formate cleavage pathways [43]. For activated sludge, the microbes retrieved in large amounts were identified as Uncultured *Enterobacter* sp. clone LSSR31 (band A3), Uncultured *Klebsiella* sp. clone FC84 (band A14), and *Bacillus* sp. AB5283 (band A22). For anaerobic granular sludge, the abundant species were Uncultured *Citrobacter* sp. clone LR244 (band G7), *Klebsiella oxytoca* strain 2-30 (band G14), and *Thermoanaerobacterium* sp. PO-2009 (band G17). Due to large amounts of these species, they might be important participants for H₂ production, substrate degradation, and soluble metabolites formation.

Based on the sequencing results, we found that the microbial community composition was similar for the two seed sludges, both performing the dominance of facultative anaerobes in microbial community. The high microbial community similarity for the two seed sludges might be due to the microbial responses during the utilization process of complex corn stover hydrolyzate. That is, the composition characteristics of corn stover hydrolyzate might cause similar selective effort on microbial species in the two seed sludges. The dominance of facultative anaerobes in microbial community could be explained as follows: since inoculum pretreatment was not applied in this study for maintaining original microbial components in the seed sludges, facultative anaerobes without spore-forming ability, such as *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp., had a good opportunity to survive from the harsh pretreatment conditions and then proliferated in the following fermentation process. Due to efficient substrate utilization and fast growth rate, facultative anaerobes rapidly dominated the microbial community during the fermentation process. Strict anaerobes, such as *Clostridium* spp., presented in small numbers and failed to compete with facultative anaerobes.

According to the H₂-producing performances in Fig. 1, we could correspondingly recognize important functional consortium for H₂ production. We found that high H₂ yields were

closely accompanied with the appearance of some strict and facultative anaerobes, which had been well investigated as efficient H₂-producing microbes while using various complex substrates. For activated sludge, the facultative anaerobe

Table 3 – Phylogenetic affiliation of 16S rDNA gene sequences from DGGE bands of activated sludge.

Band	The most similar sequence (GenBank number)	Identity
A1	Uncultured <i>Enterobacter</i> sp. clone LR191 (HM597938.1)	99%
A2	Uncultured <i>Pantoea</i> sp. clone LSSR82 (HM597952.1)	99%
A3	Uncultured <i>Enterobacter</i> sp. clone LSSR31 (HM597964.1)	99%
A4	<i>Enterobacter ludwigii</i> isolate B2 (FR752805.1)	99%
A5	<i>Enterobacter asburiae</i> strain YMC/KN/07/05 (HQ215202.1)	96%
A6	<i>Klebsiella</i> sp. bk_20 (HQ538674.1)	99%
A7	<i>Enterobacter</i> sp. RP1p (EF585402.1)	100%
A8	<i>Leclercia adecarboxylata</i> strain C107 (HQ407282.1)	99%
A9	<i>Citrobacter werkmanii</i> strain 2C1-9 (GU169032.1)	98%
A10	Uncultured <i>Citrobacter</i> sp. clone LR244 (HM597945.1)	99%
A11	<i>Enterobacter aerogenes</i> strain LCR83 (FJ976592.1)	99%
A12	uncultured gamma proteobacterium clone LL1 (AJ581650.1)	100%
A13	Uncultured <i>Serratia</i> sp. clone F7may2.72 (GQ416511.1)	100%
A14	Uncultured <i>Klebsiella</i> sp. clone FC84 (HM598006.1)	100%
A15	<i>Salmonella paratyphi</i> strain A6 (EU118081.1)	99%
A16	<i>Klebsiella oxytoca</i> strain 2-30 (GU586149.1)	100%
A17	Uncultured <i>Pectobacterium</i> sp. clone FC81 (HM598005.1)	100%
A18	<i>Leuconostoc pseudomesenteroides</i> strain 1-2 (HQ825325.1)	92%
A19	<i>Escherichia vulneris</i> strain M3 (HQ259947.1)	100%
A20	Uncultured <i>Lentisphaerae</i> bacterium 283ZH04 (GQ451984.1)	95%
A21	<i>Mycobacteriaceae</i> bacterium WR054 (AB298730.2)	94%
A22	<i>Bacillus</i> sp. AB5283 (GU366039.1)	99%

Table 4 – Phylogenetic affiliation of 16S rDNA gene sequences from DGGE bands of anaerobic granular sludge.

Band	The most similar sequence (GenBank number)	Identity
G1	<i>Enterobacter asburiae</i> strain YMC/KN/07/05 (HQ215202.1)	96%
G2	<i>Klebsiella</i> sp. bk_20 (HQ538674.1)	99%
G3	<i>Leclercia adecarboxylata</i> strain C107 (HQ407282.1)	99%
G4	<i>Citrobacter freundii</i> strain FUA1259 (HQ694731.1)	100%
G5	Uncultured <i>Klebsiella</i> sp. clone rot468 (EF645654.1)	99%
G6	<i>Citrobacter werkmanii</i> strain 2C1-9 (GU169032.1)	98%
G7	Uncultured <i>Citrobacter</i> sp. clone LR244 (HM597945.1)	99%
G8	uncultured gamma proteobacterium clone LL1 (AJ581650.1)	100%
G9	Uncultured <i>Serratia</i> sp. clone F7may2.72 (GQ416511.1)	100%
G10	<i>Kluyvera</i> sp. bk_32 (HQ538675.1)	100%
G11	Uncultured <i>Klebsiella</i> sp. clone FC84 (HM598006.1)	100%
G12	<i>Clostridium bifermentans</i> strain MKA 5 (HQ013321.1)	96%
G13	<i>Salmonella paratyphi</i> strain A6 (EU118081.1)	99%
G14	<i>Klebsiella oxytoca</i> strain 2-30 (GU586149.1)	100%
G15	Uncultured <i>Pectobacterium</i> sp. clone FC81 (HM598005.1)	100%
G16	<i>Leuconostoc pseudomesenteroides</i> strain 1-2 (HQ825325.1)	92%
G17	<i>Thermoanaerobacterium</i> sp. PO-2009 (FM999998.1)	100%

Bacillus sp. AB5283 presented large amounts at fermentation temperature of 55 °C. In a previous work, Kotay et al. [47] isolated *Bacillus coagulans* strain IIT-BT S1 from digested activated sewage sludge, and the strain reached a high H₂ yield by using glucose. It suggested that *Bacillus* sp. AB5283 might be a key H₂ producer with activated sludge as the seed sludge. For anaerobic granular sludge, the predominance of *Thermoanaerobacterium* sp. PO-2009 was observed at 55 °C, and the same strain was previously detected by Karadag et al. [46]

while performing optimal H₂ production at 60 °C. It has been reported that many efficient H₂-producing strains of *Thermoanaerobacterium* spp. have the optimal growth temperature around 60 °C, and most of them were capable of degrading complex carbohydrates along with abundant H₂ emission [13,48]. Hence, with anaerobic granular sludge as the seed sludge, *Thermoanaerobacterium* sp. PO-2009 might be mainly responsible for the better H₂-producing performance under thermophilic condition (55 °C).

Overall performances

Table 5 shows overall performances for OD₆₀₀, final pH, substrate utilization, H₂ yield, and average H₂ production rate. Among various temperatures, the fermentation process at 55 °C produced less biomass with the OD₆₀₀ of 0.71 for activated sludge and 0.75 for anaerobic granular sludge, suggesting that more substrate can be efficiently utilized for producing H₂ rather than biosynthesis. The fermentation process at 55 °C also resulted in the lowest final pH (activated sludge: 4.56; anaerobic granular sludge: 4.09), which can be attributed to more acetate production in these cases. The substrate utilization under different temperatures was all around 90%, indicating that there might still existed some complex compounds in corn stover hydrolyzate non-biodegradable for microbes under various temperatures. For the two typical seed sludges, the fermentation at 55 °C both reached the highest H₂ yield and average H₂ production rate (activated sludge: 6.08 mmol-H₂/g-utilized sugar and 0.98 mmol-H₂/(L·h); anaerobic granular sludge: 7.74 mmol-H₂/g-utilized sugar and 1.28 mmol-H₂/(L·h)).

The maximum H₂ yields and average H₂ production rates in this study were compared with previous studies (Table 6). According to the table, many studies were carried out under mesophilic conditions (35–37 °C) for H₂ production from straw hydrolyzate. While comparing H₂ yields under different temperatures, thermophilic (55 °C) and extreme thermophilic (70 °C) conditions led to relatively higher H₂ yields than mesophilic conditions, suggesting that thermophilic microbial community might be favorable for H₂ production by utilizing complex hydrolyzate. In this study, the maximum H₂ yields were slightly lower than those of comparable results under thermophilic and extreme-thermophilic conditions. The reason might be mainly due to the dominance of facultative anaerobes (*Enterobacter* spp., etc.) in microbial community,

Table 5 – Overall performances for OD₆₀₀, final pH, substrate utilization, H₂ yield, and average H₂ production rate.

Seed sludge	Temperature (°C)	OD ₆₀₀	Final pH	Substrate utilization (%)	H ₂ yield (mmol-H ₂ /g-utilized sugar)	Average H ₂ production rate (mmol-H ₂ /(L·h))
Activated sludge	37	1.04	5.29	91.3%	2.70	0.48
	30	1.49	5.48	92.8%	2.65	0.51
	55	0.71	4.56	89.8%	6.08	0.98
	70	0.78	4.99	86.4%	4.10	0.63
Anaerobic granular sludge	37	1.14	5.53	87.7%	2.24	0.82
	30	1.12	5.36	90.2%	2.43	0.91
	55	0.75	4.09	92.4%	7.74	1.28
	70	1.32	4.78	89.3%	4.58	0.73

Values represent the means of three incubations.

Table 6 – Comparison of H₂ yield and average H₂ production rate with previous studies.

Inoculum	Substrate	Temp. (°C)	H ₂ yield (mmol-H ₂ /g-utilized sugar)	Average H ₂ production rate (mmol-H ₂ /(L·h))	Ref.
Cow dung compost	Wheat straw hydrolyzate	36	3.04	0.60	[49]
Dairy manure	Corn cob hydrolyzate	36	4.82	1.88	[50]
Activated sludge	Rice straw hydrolyzate	37	5.67	n.d.	[51]
Anaerobically digested sludge	Rice straw hydrolyzate	35	1.25	n.d.	[52]
Sludge from an anaerobic digester	Wheat straw hydrolyzate	37	3.86	n.d.	[53]
Anaerobic digester Sludge	Wheat straw hydrolyzate	35	2.63	n.d.	[54]
Sludge from a local winery	Corn stover hydrolyzate	55	8.13	0.38	[55]
Anaerobic sludge	Ground wheat hydrolyzate	55	8.54	0.66	[24]
Granular sludge	Wheat straw hydrolyzate	70	9.46	1.53	[56]
Activated sludge	Corn stover hydrolysate	55	6.08	0.98	This study
Anaerobic granular sludge	Corn stover hydrolysate	55	7.74	1.28	This study

Some values of H₂ yield and production rate are calculated from literature data.
n.d.: not determined.

which always have relatively lower H₂-producing ability than strict microbes (*Clostridium* spp., etc.).

Conclusions

With activated sludge and anaerobic granular sludge as the seed sludge, mesophilic (37 and 30 °C), thermophilic (55 °C) and extreme thermophilic (70 °C) conditions were compared for their effectiveness in improving H₂ production from corn stover hydrolyzate. Experimental results indicated that fermentation temperature caused similar effects on H₂ production for the two seed sludges with the optimum H₂ yields both achieved at 55 °C (activated sludge: 6.08 mmol-H₂/g-utilized sugar; anaerobic granular sludge: 7.74 mmol-H₂/g-utilized sugar). H₂ production at 70 °C was remarkably less than at 55 °C, and fermentation processes at 37 °C and 30 °C resulted in even lower H₂ production. PCR-DGGE and corresponding biodiversity analysis indicated that fermentation temperature caused significant influences on microbial components. Generally, the higher the fermentation temperature, the lower the microbial community diversity. For the two seed sludges, facultative anaerobes, such as *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp., were dominant in microbial community. For the fermentation at 55 °C, *Bacillus* sp. AB5283 and *Thermoanaerobacterium* sp. PO-2009 might be key H₂ producers in activated sludge and anaerobic granular sludge, respectively. Among various temperatures, thermophilic condition (55 °C) was preferable for biohydrogen production from corn stover hydrolyzate.

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