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Fermentative Biohydrogen Production with Enteric Bacteria Isolated from the Intestine of Wild Common Carp Dwelling in Tarim River Basin

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Abstract

The biological hydrogen generating from fermentation of low-cost lignocellulosic feedstocks by hydrogen-producing bacteria has attracted many attentions in recent years. In the present investigation, ten hydrogen-producing bacteria were newly isolated from the intestine of wild common carp (*Cyprinus carpio* L.), and identified belonging to the genera of *Enterobacter* and *Klebsiella* based on analysis of the 16S rDNA gene sequence and examination of the physiological and biochemical characteristics. All the isolates inherently owned the ability to metabolize xylose especially the cotton stalk hydrolysate for hydrogen production with hydrogen yield (HY) higher than 100 $mL \cdot L^{-1}$. In particular, two isolates, WL1306 and WL1305 obtained higher HY, hydrogen production rate (HPR), and hydrogen production potential (HPP) using cotton stalk hydrolysate as sugar substrate than the mixed sugar of glucose & xylose, which obtained the HY of 249.5 ± 29.0, 397.0 ± 36.7 $mL \cdot L^{-1}$, HPR of 10.4 ± 1.2, 16.5 ± 1.5 $mL \cdot L^{-1} \cdot h^{-1}$, HPP of 19.5 ± 2.3, 31.0 ± 2.8 $mL \cdot L^{-1} \cdot g^{-1}_{sugar}$, separately. The generation of soluble metabolites, such as the lactate, formate, acetate, succinate and ethanol reflected the mixed acid fermentation properties of the hydrogen production pathway.

Keywords

Fermentative Biohydrogen Production, Enteric Bacteria, Intestine, Cotton Stalk Hydrolysate, Wild Common Carp

1. Introduction

Hydrogen is a clean and efficient energy with zero emission, which can be gen-

erated from utilization of various renewable feedstocks and wastes [1] [2] [3] [4]. By using carbohydrate-rich biomass, biohydrogen production can be obtained by anaerobic (dark fermentation) and photoheterotrophic (light fermentation) microorganisms [1] [2]. In recent years, biological hydrogen produced by fermentative bacteria through dark fermentation using lignocellulose as substrate has attracted many attentions [5] [6] [7] [8] [9]. Especially, the lignocellulose hydrolysate and fermentative bacteria play important roles in lignocellulose-based hydrogen production.

Cotton stalk is the most widely distributed feedstock in Xinjiang, China. In recent years, high-value utilization of cotton stalk has become a noticeable research focus. Converting the reducing sugar in cotton stalk hydrolysate into high-value chemicals has been of interests; the latest progresses were productions of bioethanol, xylitol and single cell lipid from fermentation of cotton stalk hydrolysate by different fermentative microorganisms [10] [11] [12]. Concerning to biohydrogen production, there are only two reports mentioned about the hydrogen production dynamics using cotton stalk hydrolysate as fermentative substrate [13] [14]. As such, acquirement of high-effective bacteria for efficient hydrogen production and cotton stalk hydrolysate utilization will be of great importance.

Nowadays, exploitation of high-efficiency hydrogen-producing bacteria is vitally important for the hydrogen energy development. The hydrogen-producing bacteria distributed in natural environment are with great diversity, owning to multiple metabolic pathways of hydrogen production [15]. Anaerobic fermentation is regarded as an efficient hydrogen production way with the highest hydrogen generation rate. Anaerobic fermentation of hydrogen is performed by many fermentative microorganisms, including facultative anaerobes of the *Enterobacter* genus [16] [17], anaerobes of the *Clostridium* genus [18] [19], *Methanogens* [20] and *Citrobacter* species [21]. Amongst, facultative anaerobes of the *Enterobacter* genus are the most studied hydrogen-producing bacteria, which can produce hydrogen via the formate-hydrogen lytic reaction in the mixed acid fermentation pathway [22] [23].

With the aim to acquire efficient microorganisms for hydrogen production, some hydrogen-producing bacteria have been reported to be isolated from diverse environment, such as sludge [24] [25], waste water [26], soil [27], and so on. It was proved that the hydrogen-producing bacteria isolated from unique environment may obtain good hydrogen production potential as well as specific substrate utilization property. Taguchi *et al.* (1993) reported a hydrogen-producing bacterium, *Clostridium beijerinckii* strain AM21B, which was isolated from termites, could utilize starch and glucose as substrate for hydrogen production [28]. A hydrogen-producing bacterium *Pseudomonas stutzeri* JX442762, which was isolated from thermal soil at Mettur power station, Salem district, Tamil Nadu, India, was reported to be able to use effluent as a good source for the hydrogen production with a yield of 190.03 ± 0.81 mL hydrogen [29]. As is well-known, the fish intestine is a specific environment for diverse microorganisms inhabit-

ing; most studies conducted concerning to the microbial diversity of the fish intestine and the correlation with the host development, physiology, and health [30] [31] [32] up to now, few relating to the exploitation of other functional microorganisms like hydrogen-producing bacteria.

The present study deals with isolation of hydrogen-producing bacteria from the intestine of wild common carp (*Cyprinus carpio* L.) dwelling in Tarim River Basin, Xinjiang, China. The isolates were identified by phylogenetic analysis of the 16S rDNA sequence and examination of the physiological and biochemical characteristics. The hydrogen production properties of the isolates using various sugar substrates were examined to obtain the strains capable of utilizing cotton stalk hydrolysate for hydrogen production. The soluble metabolites generated during the hydrogen production process using cotton stalk hydrolysate as sugar substrate were analyzed to convince the hydrogen production metabolic pathway of the isolated strains.

2. Materials and Methods

2.1. Materials

The wild common carp (*Cyprinus carpio* L.) was caught from Tarim River Basin, the live fish was surface-sterilized and slit the belly soon after being caught, and the intestines were then pulled out and immediately prepared for isolation of hydrogen-producing bacteria.

The cotton stalks used in this research were harvested from a cotton field in Xinjiang Alaer, China. The stalks were dried, milled into fragments, and sifted using a 20-mesh sieve before hydrolysis. The cotton stalk hydrolysate was obtained using the optimum hydrolysis technology and detoxification & decolorization methods according to the previous report [10] [11] [12]. As a result, the hydrolysate was composed mainly of glucose and xylose, whose concentration ratio is about 3:1 [12], which was prepared as constituent of the fermentation medium.

2.2. Isolation of Hydrogen-Producing Bacteria Capable of Utilizing Cotton Stalk Hydrolysate

1 g of the intestines and inclusions were weighed and added into 9 mL sterilized water, then were blended and ground to be the initial suspension, which was then diluted ten times serially. The suspensions diluted 10^5, 10^6, 10^7 times were transferred with 0.1 mL into sterile isolation plate and spread evenly, and incubated in a constant-temperature incubator at 37˚C for 48 h. The isolation medium contained: beef extract 5 g/L, peptone 10 g/L, NaCl 5 g/L, agar 20 g/L. After incubation, the single colonies were selected and inoculated into 18 mL tube with 10 mL liquid medium (the preliminary screening medium included glucose 10 g/L, xylose 10 g/L, peptone 10 g/L, beef extract 5 g/L, NaCl 5 g/L; the secondary screening medium added cotton stalk hydrolysate with reducing sugar concentration of 20 g/L, peptone 10 g/L, beef extract 5 g/L, NaCl 5 g/L), and the

Durham's fermentation tube immersed under the liquid level to collect gas produced by the isolates, the inoculums were cultured at 37˚C for 24 h. The isolates that could grow and produce H_2 gas in the preliminary screening medium were selected to inoculate into the secondary screening medium, and those could grow and produce H_2 concentration higher than 50 mL/L_{medium} were considered as hydrogen-producing isolates.

2.3. Identification of the Isolated Strains

Genomic DNA was extracted from hydrogen-producing bacteria cells in the exponential phase using an Ezup Column Bacteria Genomic DNA Purification Kit (Sangon, China) in the guidance of the manufacturer's instructions. The 16S rDNA genes were amplified by PCR using the primer pair 27F/1492R. The PCR products were sequenced, and the 16S rDNA sequences were aligned and identified against existing sequences in the GenBank database using the BLAST program. Further, the nucleotide sequences of the isolates were aligned with closely related sequence using clustal W program of Mega software (version 6.0) and a phylogenetic tree was constructed to show the relationship between the isolates and the reference strains.

Gram staining of the bacteria was performed using the Hucker method, which was previously reported by Doetsch [33]. The bacterial morphologies were examined using an optical microscope DM1000 LED (Leica, Germany). The bacterial physiological and biochemical characteristics were examined according to the protocols described in the Identification Manual of Systematic Bacteriology [34].

2.4. Hydrogen Production from Fermentation of Various Sugar Media

The isolates were cultured on activation slants for 24 h, three loopfuls of activated cells were inoculated into a 250 mL Erlenmeyer flask containing 100 mL of seed medium and incubated at 37˚C on a rotary shaker at 150 rpm within 16 h. The seed medium contained: glucose 10 g/L, xylose 10 g/L, peptone 10 g/L, beef extract 5 g/L, NaCl 5 g/L. OD_{600}s of the seeds were modulated to 1.0 approximately, and inoculated into the fermentation medium with inoculation size of 10% (v/v). For each fermentation sample, 175 mL of fermentation medium was loaded into a 250 mL Erlenmeyer flask and incubated in a constant-temperature incubator at 37˚C for 24 h. A rubber plug with a pipe was used for each flask in order to seal the flask and transfer gas produced by each isolate, the fermenter is illustrated as **Figure 1**. The fermentative media with various sugar were designed as follows: glucose 20 g/L (it could be replaced using other designed sugars: 1) xylose 20 g/L; 2) glucose 10 g/L & xylose 10 g/L; 3) cotton stalk hydrolysate with reducing sugar concentration of 20 g/L), beef extract 5 g/L, peptone 10 g/L, NaCl 5 g/L, KH_2PO_4 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L. After fermentation, the volume and concentration of hydrogen gas were measured, and the sugar content in the fermentative broth was examined. In the treatment of using cotton stalk hydrolysate

Figure 1. The fermenter for biohydrogen production.

as sugar substrate, the main by-products such as succinate, citrate, lactate, acetate, ethanol, were also examined.

2.5. Analytical Methods

Volume of the hydrogen gas was measured by 1 mol/L NaOH displacement in an inverted burette, and a handled hydrogen detector (KP810H20, Henan Zhong'an Electronic Detection Technology Co. Ltd., China) was used to examine the biohydrogen concentration. At end of the fermentation, the aqueous samples were centrifuged at 8000 × g for 10 min and filtered through syringe filters with 0.22 μm membrane before being analyzed.

The total concentration of reducing sugars in the broth was determined by the 3,5-dinitryl-salicylic acid reagent (DNS) method reported previously [35]. Glucose and xylose concentrations were detected by high-performance liquid chromatograph (HPLC) (Shimadzu LC-2A) using a refractive index detector. A Cosmosil NH_2 column (5 μm, 4.6 mm × 250 mm) was used with a solution of acetonitrile and water (75:25) as the eluent. Analysis was developed using an eluent flow rate of 1.0 mL/min at a temperature of 40˚C, and the injection volume of 20 μL.

The concentrations of succinate, citrate and lactate were measured by a high-performance liquid chromatograph (HPLC) (Waters2695) with a UV-detector and using a C_{18} silica gel column Sinochrom ODS–BP (4.6 mm × 250 mm × 5 μm). The $NH_4H_2PO_4$ solution of 10 mmol/L was used as mobile phase with a flow rate of 1.0 mL/min, the temperature of column was 37˚C, the detection wavelength was 210 nm, and the injection volume was 20 μL. The concentrations of

acetate and ethanol were verified by gas chromatography (GC) (Aglient6890N, J&W Scientific) with a flow rate of 2 mL/min over the hp-FFAP column (30 m × 0.25 mm × 0.25 μm) using flame ionization detector (FID) detector with a nitrogen carrier gas. The process conditions were conducted as follows: injector temperature, 220˚C; detector temperature 280˚C; the temperature profile 60, 170˚C with 6 min run time.

3. Results and Discussion

3.1. Isolation and Identification of the Hydrogen-Producing Bacteria

The intestine of wild common carp (*Cyprinus carpio* L.) is a comfortable habitat for many fermentative bacteria. More than 50 bacteria isolated from the isolation medium and purified for follow-up experiment. Among them, 22 bacteria were selected as candidate strains with the ability to grow and produce H_2 gas in the preliminary screening medium. On the basis, 10 bacteria, which obtained OD_{600} of growth and concentration of hydrogen production higher than 1.0 and 50.0 mL/L_{medium} separately in 24-hour culture, were picked out as the aimed hydrogen-producing isolates (Figure 2). All the ten bacteria were designated with numbers of WL1306, WL1315, WL1302, WL1307, WL1318, WL1308, WL1305, WL1310, WL1309, WL1312. All the bacteria are rod-shaped and Gram-staining negative. Physiological and biochemical characteristics of the isolates were also examined, and the results were illustrated in Table 1, which were similar to properties belonging to the genera of *Enterobacter* and *Klebsiella*.

Furthermore, the bacterial sequences of 16S rDNA genes (approximately 1.5 kb) were sequenced and then submitted to the GenBank to obtain the accession numbers of KT328451, KT328457, KT328449, KT328452, KT328458, KT328453, KT328450, KT328455, KT328454, KT328456. The determined sequences were compared with the available 16S rDNA gene sequences from the GenBank database by the BLAST search program, and a phylogenetic tree was constructed to show the relationship between isolates deduced from the determined sequences and the reference bacterial strains (Figure 3). It was deduced from Figure 3 that the isolates could be divided into two genera of *Enterobacter* and *Klebsiella*, amongst, five species, such as WL1310, WL1302, WL1318, WL1306, WL1308,

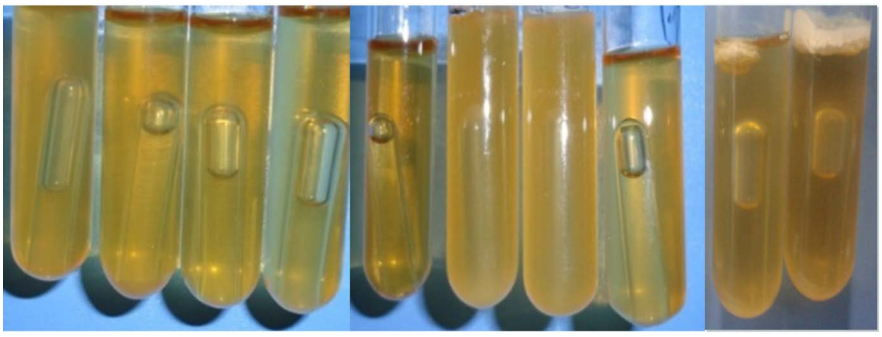

Figure 2. Hydrogen gas generation in screening medium.

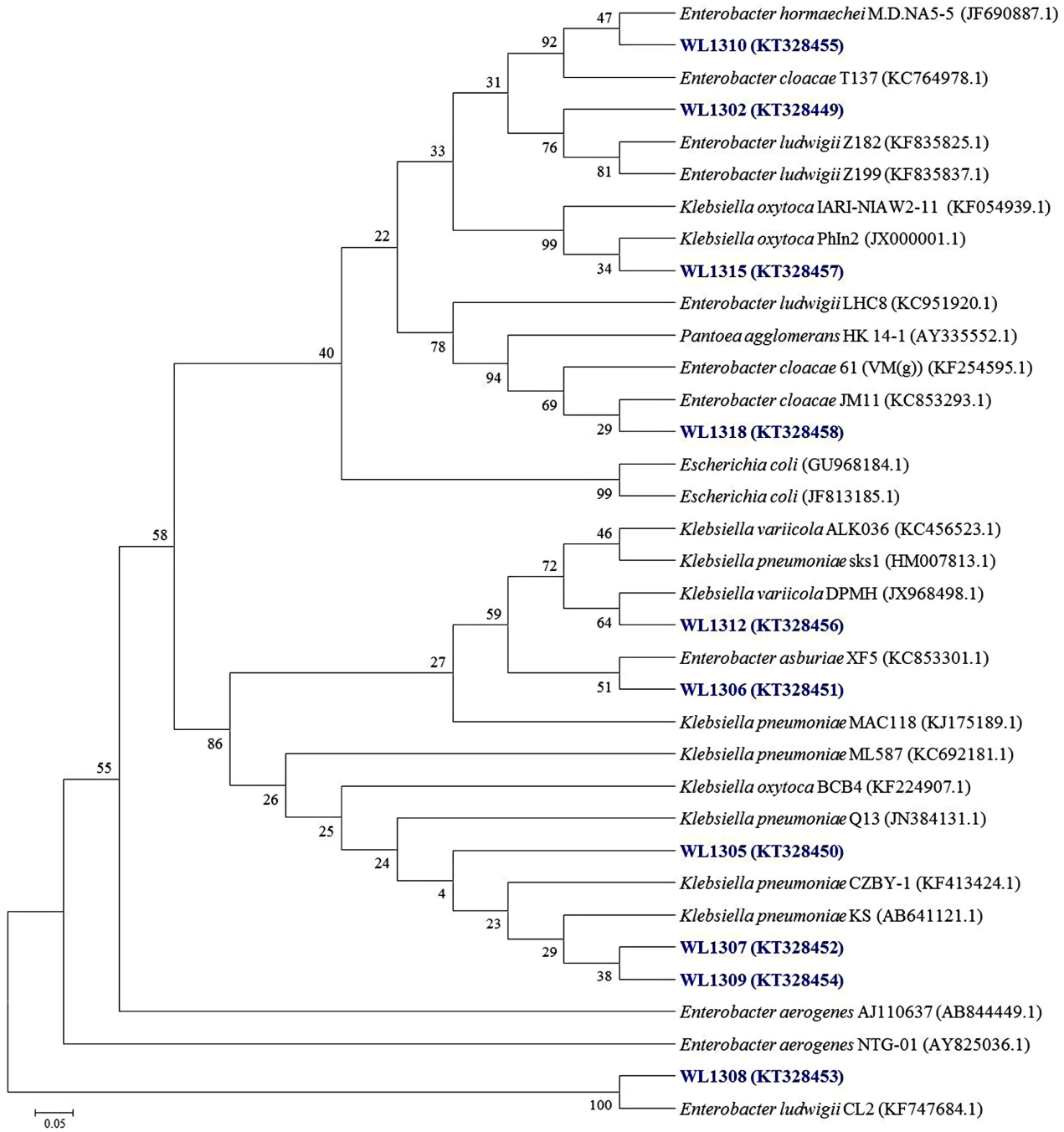

Figure 3. Neighbour-joining phylogenetic tree showing the phylogenetic position of the isolates, based on 16S rDNA gene sequences of *Enterobacteriaceae*. The numbers at the nodes indicate the levels of bootstrap support based on the neighbor-joining analysis of 1000 resampled data sets. Bar 0.05 substitutions per nucleotide position.

were related to *Enterobacter* genus. On the other hand, five species, such as WL1315, WL1312, WL1305, WL1307, WL1309, were grouped into *Klebsiella* genus. Species in genera of *Enterobacter* and *Klebsiella* are the mostly reported facultative anaerobic hydrogen-producing bacteria, which can utilize various substrates for hydrogen production via mix acid pathway [36] [37] [38] [39]. The dominant hydrogen-producing bacteria isolated from the intestine of wild

Table 1. Physiological and biochemical characteristics of the hydrogen-producing bacteria.

Characteristics	WL 1306	WL 1315	WL 1302	WL 1307	WL 1318	WL 1308	WL 1305	WL 1310	WL 1309	WL 1312
Gram staining	−	−	−	−	−	−	−	−	−	−
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Citrate utilization	−	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+
Methyl-red test	−	+	−	+	−	−	+	−	+	−
V-P test	+	+	+	+	+	+	+	+	+	+
Production of indole	+	+	+	+	+	+	+	+	+	+
Production of H_2S	−	−	−	−	−	−	−	−	−	−
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Litmus milk	+	+	+	+	+	+	+	+	+	+
Lipase	−	−	−	−	−	−	−	−	−	−
Urease	−	−	−	−	−	−	−	−	−	−

+: positive; −: negative.

common carp were just the two types, indicating that the bacteria isolated from this unique environment might possess high hydrogen production potential.

3.2. Fermentative Hydrogen Production Properties of the Isolates

In order to make clear the hydrogen production potential of the isolates, the hydrogen producing properties using various reducing sugar were examined (**Figure 4**). Glucose is the essential carbon source for hydrogen producing bacteria, which was mentioned in many reports [40] [41] [42]. All the ten isolates could effectively produce hydrogen in the glucose medium with hydrogen yield (HY), hydrogen production rate (HPR), hydrogen production potential (HPP) higher than 100 $mL \cdot L^{-1}$, 5.5 $mL \cdot L^{-1} \cdot h^{-1}$, 10.5 $mL \cdot L^{-1} \cdot g^{-1}_{sugar}$, illustrating that all the ten isolated bacteria could utilize glucose as elementary sugar substrate for hydrogen production. Not only that, the isolates could also utilize xylose for hydrogen production. Especially, the isolates obtained higher HY, HPR and HPP using xylose as sugar substrate than glucose, indicating that the isolates inherently owned the ability to metabolize xylose for hydrogen production (**Table 2**).

Noticeably, the isolates also obtained high HY, HPR and HPP using glucose & xylose and cotton stalk hydrolysate as sugar substrate, showing their abilities to utilize glucose and xylose simultaneously for hydrogen production. Several strains, such as WL1310, WL1309, WL1312 acquired remarkably high hydrogen production with HY of 718.5 ± 9.2, 679.5 ± 4.9, 906.0 ± 8.5 $mL \cdot L^{-1}$, respectively, using glucose & xylose as sugar substrate. Moreover, all the isolates could utilize cotton stalk hydrolysate for hydrogen production with HY higher than 100 $mL \cdot L^{-1}$, particularly, two strains, WL1306 and WL1305 obtained higher HY, HPR

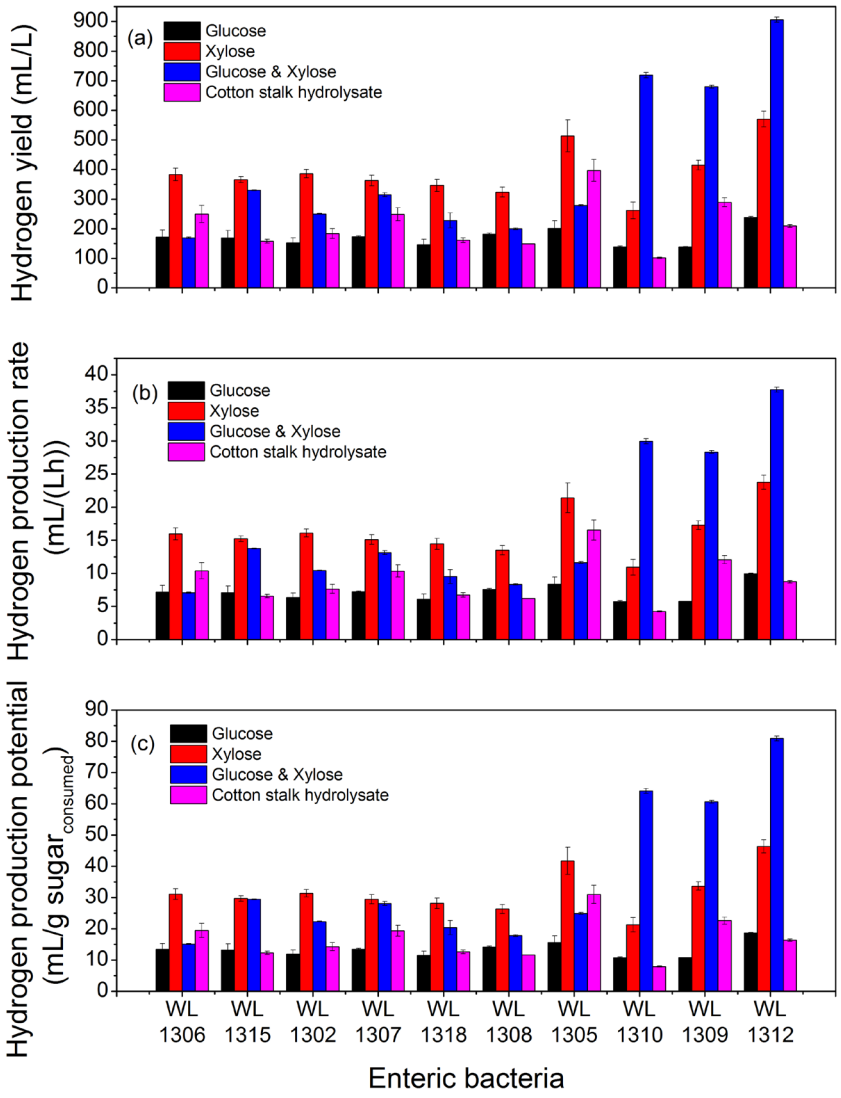

Figure 4. Hydrogen production by the hydrogen-producing bacteria from fermentation of various sugar substrate (glucose, xylose, glucose & xylose, cotton stalk hydrolysate). (a) Hydrogen yield (HY); (b) Hydrogen production rate (HPR); (c) Hydrogen production potential (HPP).

Table 2. Hydrogen production by the hydrogen-producing bacteria from fermentation of various sugar media.

Sugar substrates	Items	WL1306	WL1315	WL1302	WL1307	WL1318	WL1308	WL1305	WL1310	WL1309	WL1312
Glucose	HY ($mL \cdot L^{-1}$)	172.0 ± 24.0	169.5 ± 24.7	152.5 ± 16.3	172.5 ± 3.5	146.5 ± 17.7	181.5 ± 3.5	200.5 ± 26.2	138.0 ± 2.8	138.5 ± 0.7	238.5 ± 2.1
	HPR ($mL \cdot L^{-1} \cdot h^{-1}$)	7.2 ± 1.0	7.1 ± 1.0	6.4 ± 0.6	7.2 ± 0.1	6.1 ± 0.7	7.6 ± 0.1	8.4 ± 1.0	5.8 ± 0.1	5.8 ± 0.1	9.9 ± 0.1
	HPP ($mL \cdot L^{-1} \cdot g^{-1}_{sugar}$)	13.4 ± 1.8	13.2 ± 1.9	11.9 ± 1.3	13.5 ± 0.3	11.4 ± 1.3	14.2 ± 0.3	15.7 ± 2.0	10.8 ± 0.2	10.8 ± 0.1	18.6 ± 0.2
Xylose	HY ($mL \cdot L^{-1}$)	383.0 ± 21.2	365.5 ± 10.6	386.0 ± 14.1	362.5 ± 17.7	346.5 ± 20.5	324.0 ± 17.0	514.0 ± 53.7	262.0 ± 28.3	414.5 ± 16.3	570.5 ± 26.2
	HPR ($mL \cdot L^{-1} \cdot h^{-1}$)	16.0 ± 0.9	15.2 ± 0.4	16.1 ± 0.6	15.1 ± 0.7	14.4 ± 0.9	13.5 ± 0.7	21.4 ± 2.2	10.9 ± 1.2	17.3 ± 0.7	23.7 ± 1.1

Continued

Glucose & Xylose	HPP ($mL \cdot L^{-1} \cdot g^{-1}_{sugar}$)	31.1 ± 1.7	29.7 ± 0.9	31.4 ± 1.1	29.5 ± 1.4	28.2 ± 1.7	26.3 ± 1.4	41.8 ± 4.3	21.3 ± 2.3	33.7 ± 1.3	46.3 ± 2.1
	HY ($mL \cdot L^{-1}$)	169.5 ± 2.1	330.0 ± 1.4	250.0 ± 1.4	315.0 ± 7.1	228.0 ± 25.5	200.0 ± 2.8	279.0 ± 2.8	718.5 ± 9.2	679.5 ± 4.9	906.0 ± 8.5
	HPR ($mL \cdot L^{-1} \cdot h^{-1}$)	7.1 ± 0.1	13.8 ± 0.1	10.4 ± 0.1	13.1 ± 0.3	9.5 ± 1.0	8.3 ± 0.1	11.6 ± 0.1	29.9 ± 0.4	28.3 ± 0.2	37.8 ± 0.4
	HPP ($mL \cdot L^{-1} \cdot g^{-1}_{sugar}$)	15.1 ± 0.1	29.5 ± 0.1	22.3 ± 0.1	28.1 ± 0.6	20.4 ± 2.1	17.9 ± 0.3	24.9 ± 0.3	64.2 ± 0.8	60.7 ± 0.5	80.9 ± 0.8
Cotton stalk hydrolysate	HY ($mL \cdot L^{-1}$)	249.5 ± 29.0	157.5 ± 6.4	183.5 ± 16.3	248.5 ± 61.5	161.5 ± 7.8	149.0 ± 0.0	397.0 ± 36.7	101.5 ± 2.1	289.5 ± 14.8	209.5 ± 4.9
	HPR ($mL \cdot L^{-1} \cdot h^{-1}$)	10.4 ± 1.2	6.6 ± 0.3	7.6 ± 0.7	10.4 ± 0.9	6.7 ± 0.3	6.2 ± 0.0	16.5 ± 1.5	4.2 ± 0.1	12.1 ± 0.6	8.7 ± 0.2
	HPP ($mL \cdot L^{-1} \cdot g^{-1}_{sugar}$)	19.5 ± 2.3	12.3 ± 0.5	14.3 ± 1.2	19.4 ± 1.7	12.6 ± 0.6	11.6 ± 0.0	31.0 ± 2.8	7.9 ± 0.2	22.6 ± 1.1	16.6 ± 0.5

HY: hydrogen yield; HPR: hydrogen production rate; HPP: hydrogen production potential.

and HPP using cotton stalk hydrolysate as sugar substrate than the mixed sugar of glucose & xylose, which obtained the HY of 249.5 ± 29.0, 397.0 ± 36.7 $mL \cdot L^{-1}$, HPR of 10.4 ± 1.2, 16.5 ± 1.5 $mL \cdot L^{-1} \cdot h^{-1}$, HPP of 19.5 ± 2.3, 31.0 ± 2.8 $mL \cdot L^{-1} \cdot g^{-1}_{sugar}$, separately, illuminating that the two bacteria might be more liable to utilize cotton stalk hydrolysate for hydrogen production.

3.3. Soluble Metabolites Analysis of Hydrogen Production from Cotton Stalk Hydrolysate

The ten isolates exhibited different hydrogen production performance when using cotton stalk hydrolysate as sugar substrate as shown in the above section, indicating that the hydrogen production properties might be different. So analysis of soluble metabolites generated during the hydrogen production process would be necessary. *Enterobacter* and *Klebsiella* species are known to carry out mixed acid fermentation while using sugars as the carbon substrate [36] [37]. In the present work, the soluble metabolites produced from the enteric bacteria during dark H_2 fermentation were lactate, formate, acetate, succinate and ethanol (**Figure 5**). Production of lactate was predominant in the isolates of WL1307 and WL1309, accounting for 45.4% (w/w) and 57.0% (w/w) of total soluble metabolites formation in each strain. While production of succinate was predominant in the strains of WL1308 and WL1312, accounting for 48.3% (w/w) and 42.3% (w/w) of total soluble metabolites formation in each strain. The ethanol produced at a comparable high concentration in strains WL1305, WL1309, WL1315, WL1307, which obtained ethanol concentration higher than 0.65 g/L. Moreover, acetate production was apparently not the preferable metabolic pathway for the enteric bacteria, since the contribution of acetate to soluble metabolites was, in general, less than 25% of the total soluble metabolites, especially, the acetate concentrations, produced by WL1308 and WL1312, were almost close to 0 g/L. Although the formate concentrations were higher than acetate in every strains,

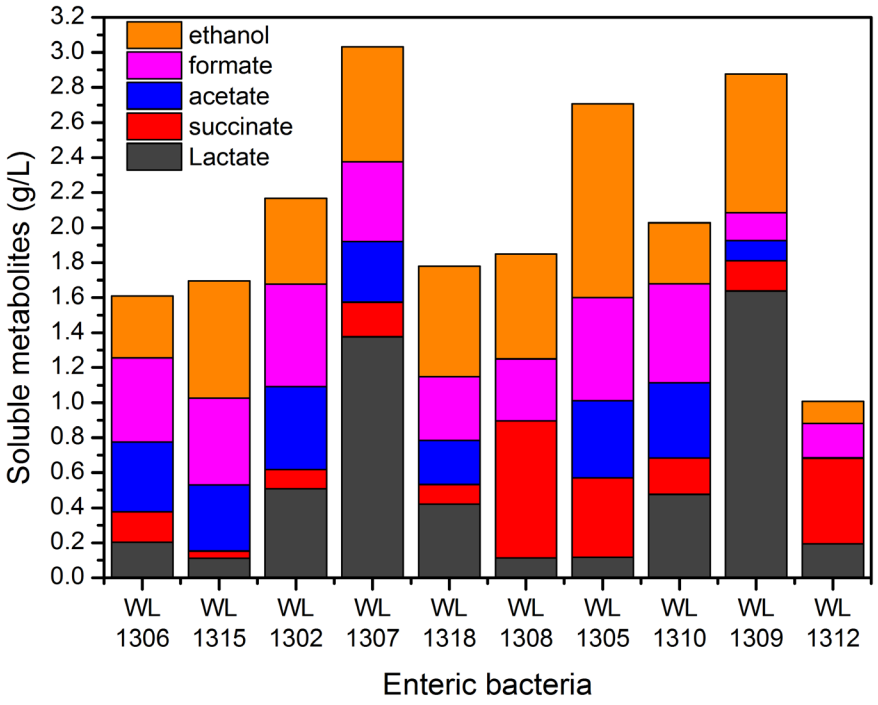

Figure 5. The soluble metabolites produced from the enteric bacteria during the hydrogen production process using cotton stalk hydrolysate as substrate.

the proportion of formate in the total soluble metabolites was still low, implying that the isolates producing hydrogen through formate hydrogen lyse pathway may affected by other metabolites generating branch pathway. In contrast, the soluble metabolite composition resulting from *Enterobacter* genus was very different from that for the *Klebsiella* genus. *Klebsiella* species are common alcohol producers and frequently used for the production of commercially valuable alcohol [43]. Indeed, the major product for *Klebsiella* sp. during dark H_2 fermentation was ethanol, while smaller quantities of aecetate and formate were formed in most *Klebsiella* species.

4. Conclusion

Ten hydrogen-producing bacteria WL1306, WL1315, WL1302, WL1307, WL1318, WL1308, WL1305, WL1310, WL1309, WL1312, newly isolated from the intestine of wild common carp (*Cyprinus carpio* L.), which were rod-shaped and Gram-staining negative strains belonging to the genera of *Enterobacter* and *Klebsiella* based on the 16S rDNA gene sequence analysis and examination of the physiological and biochemical characteristics. All the ten hydrogen-producing bacteria were capable of producing hydrogen gas in the media using glucose, xylose, glucose & xylose and cotton stalk hydrolysate as sugar substrate. Especially, all the isolates obtained higher HY, HPR, HPP using xylose as sugar substrate than glucose, indicating that the isolates inherently owned the ability to metabolize xylose for hydrogen production. Moreover, all the isolates could utilize cotton stalk hydrolysate for hydrogen production with hydrogen yield (HY)

higher than 100 $mL \cdot L^{-1}$. In particular, two isolates, WL1306 and WL1305 obtained higher HY, HPR and HPP using cotton stalk hydrolysate as sugar substrate than the mixed sugar of glucose & xylose, which obtained the HY of 249.5 ± 29.0, 397.0 ± 36.7 $mL \cdot L^{-1}$, HPR of 10.4 ± 1.2, 16.5 ± 1.5 $mL \cdot L^{-1} \cdot h^{-1}$, HPP of 19.5 ± 2.3, 31.0 ± 2.8 $mL \cdot L^{-1} \cdot g^{-1}_{sugar}$, separately, illuminating that the two bacteria might be more liable to utilize cotton stalk hydrolysate for hydrogen production. The generation of soluble metabolites, such as the lactate, formate, acetate, succinate and ethanol reflected the mixed acid fermentation properties of the hydrogen production pathway. In summary, the present investigation provided an effective way to isolate the hydrogen-producing bacteria from the intestine of wild common carp (*Cyprinus carpio* L.) for fermentative biohydrogen production using cotton stalk hydrolysate as carbon source, and revealed the hydrogen production potential and the soluble metabolites generating properties of the isolates.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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