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Enhanced Biogas Production by Ligninolytic Strain *Enterobacter hormaechei* KA3 for Anaerobic Digestion of Corn Straw

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Abstract: Lignin-feeding insect gut is a natural ligninolytic microbial bank for the sustainable conversion of crop straw to biogas. However, limited studies have been done on highly efficient microbes. Here, an efficient ligninolytic strain *Enterobacter hormaechei* KA3 was isolated from the gut microbiomes of lignin-feeding *Hypomeces squamosus* Fabricius, and its effects on lignin degradation and anaerobic digestion were investigated. No research has been reported. Results showed that strain KA3 had better lignin-degrading ability for corn straw with a higher lignin-degrading rate (32.05%) and lignin peroxidase activity (585.2 U/L). Furthermore, the highest cumulative biogas yield (59.19 L/kg-VS) and methane yield (14.76 L/kg-VS) were obtained for KA3 inoculation, which increased by 20% and 31%, respectively, compared to CK. Higher removal rates of COD, TS, and vs. of 41.6%, 43.11%, and 66.59% were also found. Moreover, microbial community diversity increased as digestion time prolonged in TG, and bacteria were more diverse than archaea. The dominant genus taxon, for methanogens, was *Methanosate* in TG, while in CK was *Methanosarcina*. For bacteria, dominant taxa were similar for all groups, which were *Solibacillus* and *Clostridium*. Therefore, strain KA3 improved the methane conversion of the substrate. This study could provide a new microbial resource and practical application base for lignin degradation.

Keywords: microbial lignin degradation; gut microbiome; *Enterobacter hormaechei* KA3; biogas production; microbial community

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1. Introduction

Biogas, one candidate to replace fossil fuels, has great advantages for relieving energy shortage and environmental pollution [1,2]. Crop straw has been widely used to generate biogas in the anaerobic digestion (AD) process. In China, about 10.4 billion tons of various crop straws are produced each year, with a large methane production potential of about 50 billion m^3 [3]. However, in the crystal structure of straws, lignin is a uneven and irregular aromatic polymer surrounding cellulose and hemicellulose [3]. It is difficult to degrade, especially under anaerobic conditions, which further seriously hinders the methane conversion efficiency of crop straw [4,5]. On the other hand, the traditional physicochemical pretreatments of lignin show high energy consumption, environmental pollution, and low efficiency [6]. Therefore, finding highly efficient and pollution-free methods for lignin degradation is of great significance for the promotion of the methane conversion of agricultural wastes as well as the protection of the environment.

Microbial lignin degradation has significant advantages of environmental friendliness, high efficiency, and economic feasibility, and the energy-based reuse of metabolites can be achieved [7]. Fungi and bacteria are the main ligninolytic microbes. Most of them have been identified in different habitats, such as soil, rotten wood, activated sludge, wastewater treatment plants, compost, and animal guts. Fungi, such as *Phanerochaete Chrysosporium*,

Hypholoma fasciculare, and *Didymosphaeria* sp., show higher efficiency [8,9], but they are sensitive to environmental conditions. Bacteria have good tolerance to environmental changes, so they have good potential for industrial application of lignin degradation. The gut of lignin-feeding insects is a natural microbial resource for lignin degradation, including termites, locusts, and cerambycidae. The gut bacterial systems are diverse, with abundant symbiotic bacteria for lignin degradation [10], including *Enterobacteriaceae*, *Bacteroides*, *Staphylococcus*, *Streptococcus*, and *Bacillus*. They can degrade about 20–100% of lignin model compounds, and the ability is about 30–40% higher than that of large herbivores [11]. Termites have been widely reported as a model insect for screening lignocellulose-degrading microorganisms [12]. Although immense breakthroughs have been achieved in termite gut microbiomes, much of these ligninolytic microbes in lignin-feeding insect guts have yet to be discovered, and their uses are largely untapped.

Hypomeces squamosus Fabricius is a common pest that feeds on the tender leaves of crops in the south of China [13]. At present, the research on the *Hypomeces squamosus* Fabricius is mainly about pest control and management in agriculture and forestry. In the study of Xu F.F. et al., *Hypomeces squamosus* Fabricius was used to investigate the rate of extrafloral nectary (EFN) secretion in response to herbivore and damage types [14]. The new 'trial marriage' model for females' mate choice proposed by Li my et al., which was tested in *Hypomeces squamosus* Fabricius, explored how females exchange mates under experimentally induced interference of male mating [15]. However, studies on *Hypomeces squamosus* Fabricius gut microbiota composition and screening of highly efficient lignin-degrading strains have not been reported. This study first found *Hypomeces squamosus* Fabricius at the Xiama forest farm in Tianzhu County, Wuwei city, Gansu Province. Leaves of *Iris ensata* Thunb (*Iridacea*) with a high lignin content of 32% is their only food source. This indicated that this insect could digest and metabolize lignin. Therefore, we defined it as lignin-feeding *Hypomeces squamosus* Fabricius. No relative research has been done to date.

Consequently, this study hypothesized that highly efficient ligninolytic microbes could be contained in the gut microbiomes of lignin-feeding *Hypomeces squamosus* Fabricius, which could be used to improve the conversion rate of crop straw to produce biogas. This work is of great significance for the recycling of agricultural wastes and the promotion of the methane conversion of crop straw while mitigating pollution of the environment. To prove this, in this study, (i) an efficient ligninolytic strain was screened and its lignin-degrading ability of corn straw (CS) was measured, (ii) biogas production and the substrate conversion rate in the AD process of CS were investigated, and (iii) microbial community characteristics (bacterial and archaeal) were also analyzed.

2. Materials and Methods

2.1. Isolation and Identification of the Ligninolytic Strain

Thirty *Hypomeces squamosus* Fabricius adults were put into a sterilized EP tube and cleaned with sterilized phosphate buffer after sterilization with 75% ethanol for 3–5 min. Their guts were extracted with tweezers and collected in SW and then transferred to a crucible and ground well to obtain a gut suspension. Then, the gut suspension was transferred into Luria–Bertaini (LB) medium and incubated for 12 h. Cultures at different gradient dilution concentrations (10^{-3}, 10^{-5}, and 10^{-7}) were plated on selection medium plates at 37 °C for 48 h. Further, after the colonies grew, we chose different morphologies of colonies, and pure isolates were obtained. A purified single colony was picked up to extract the total DNA of bacteria using the TakaRa bacterial genome extraction kit, and the 16S rRNA gene of bacteria was amplified using universal primers for the 16S rRNA gene. Finally, the PCR products were sent to BGI for sequencing. LB medium contained 5.0 g yeast extract, 5.0 g peptone, and 10 g NaCl in 1 L medium. Selective medium was prepared with distilled water contained 0.2 g Na_2HPO_4, 1.0 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgSO_4$, 1.33 g NH_4NO_3, and 2.0 g lignin in 1 L medium. Saline water (SW) consisted of 8.5 g NaCl with 1 L distilled water, and the pH was adjusted to 7.2. MS medium contained 0.125 g

Na_2HPO_4 $12H_2O$, 0.45 g NaH_2PO_4, 0.234 g K_2HPO_4 $3H_2O$, 0.18 g KH_2PO_4, 0.18 g $MgSO_4$, 0.9 g (NH_4) $2SO_4$, and 0.09 g $CaCl_2$ in 1 L medium; the carbon source in the medium was added according to 1% (w/V). The above media were sterilized at 121 °C for 15 min.

2.2. Measurement of the Lignin-Degrading Ability of the Ligninolytic Strain

2.2.1. Determination of the Lignin Degradation Rate

CS was collected from Lanzhou University, Yuzhong County, Lanzhou city, Gansu Province. It was ground into a fine powder over a 60 mesh sieve and stored before the experiment. In 250 mL Erlenmeyer flasks, 100 mL of MS medium and, proportionally, 1% CS powder were added. After steam sterilization at 121 °C for 15 min, 100 µL of each culture solution of the screened strain was also added. The control did not have culture solution addition. Each group was conducted in triplicate. The lignin content of each treated culture system was determined after seven days of incubation at 37 °C on a shaker at 150 rpm [16]. Determination of lignin peroxidase and laccase activities was performed as previously described [17].

2.2.2. Scanning Electron Microscope Analysis of the Pretreated CS

Four small pieces of CS with square shape size of 1 cm per side were added to the MS medium. The screened strain was added after cooling to room temperature by steam sterilization at 121 °C for 15 min. The control group did not contain the strain. After one week of incubation on a shaker (37 °C, 150 rpm), CS pieces were removed and soaked with 2.5% pentanediol solution overnight. Then, they were sequentially soaked in ethanol at 30%, 50%, 70%, 80%, and 90% for 10 min for dehydration. The dehydrated CS was freeze-dried in a freeze dryer for 6 h. Finally, a scanning electron microscope (SEM) (s-3400, HITACHI, Tokyo, Japan, 2018) was used to observe the CS surface structure after gold spraying for 30 s.

2.3. Effects of the Ligninolytic Strain on the AD of CS

2.3.1. Substrate and Inoculum

The air-dried CS ground into the size of 2–3 mm pieces was used as the substrate, and fresh cow manure (CM) collected from a local cow farm in Yuzhong, China, was used as the inoculum. The physicochemical characteristics of CS and CM were determined prior to AD experiments as shown in Table S1 (Supplementary Materials).

2.3.2. Experimental Design

Batch laboratory-scale and controllable constant-temperature digestion equipment was used in this study; a 500 mL glass bottle was used as the digestion reactor with a 300 mL working volume. The material ratio of CM to CS was 13.9 (based on TS). The screened strain was cultured in LB medium until the OD_{600} value reached 0.6; then, 15 mL of bacterial broth was taken and centrifuged at 12,000 rpm for 10 min, washed three times with normal saline, and suspended in 10 mL of normal saline [18–20]. All of them were inoculated in the digestion reactors (treated group, TG), and no strain addition was set as the control group (CK). Before assembly, the digestion flask was subjected to a 15 s N_2 purge to remove O_2. Each digestion bottle had a silicone stopper and was connected to a gas collection bag and sampling tube. The biogas production was measured on a daily basis using a 60 mL syringe. The daily biogas production was recorded during the digestion period. The centrifugal supernatant of digested sludge was collected every three days for volatile fatty acid (VFA) and chemical oxygen demand (COD) detection, and the solids digested for seven days (7D) and 18 days (18D) were collected for microbial community analysis. All samples were conducted in triplicate under mesophilic conditions (37 ± 1 °C) for 35 days.

2.3.3. Analytical Methods

CH_4 and CO_2 contents (% biogas) were examined by gas chromatography (SP-3420A, Beijing Beifen-Ruili Company, Beijing, China, 2019) with a porapak-Q column, and a standard curve was drawn using known standard gases (CH_4 60%, CO_2 40%). Detector: TCD, temperature 100 °C and injection port temperature 100 °C. Carrier gas: Argon, 40 mL/min. VFA and acetic acid (mg/L) were also detected by the same gas chromatograph, and the detailed procedures were described previously [21]. After drying at 105 °C and burning into ash in the muffle furnace (sx2-4-10A, Tianjin, China, 2013) at 600 °C for 2 h, the total solid (TS) and volatile solid (VS) were determined by weighing. COD was measured by a COD/total organic carbon (TOC) multiparameter water meter (ET99731, Lovibond, Germany, 2020).

2.4. DNA Extraction, PCR Amplification, and Illumina Sequencing

An E.Z.N. soil DNA kit (OMEGA, Norwalk, CT, USA, 2020) was used to extract the total microbial DNA from 0.1 g of anaerobic digestion sample following the manufacturer's instructions. The concentration and quality of the DNA were measured using a spectrophotometer (NanoDrop2000, Thermo Scientific, Wilmington, DE, USA, 2019). Then, the quality-checked DNA sample was stored at −80 °C before PCR amplification and 16S sequencing. The archaeal 16S region was amplified using primers: 524F10extF: 5′-TGYCAGCCGCCGCGGTAA-3′ and Arch958RmodR: 5′-YCCGGCGTTGAVTCCAATT-3′. PCR amplification of bacterial 16S rRNA was conducted by sequencing the V3–V4 hypervariable region using the primers 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ [22]. The detailed operation is referred to in a previous report [23].

2.5. Statistical Analysis

The results of the lignin degradation rate, LiP activity, and fermentation-related indicators were expressed as the mean ± standard deviation (SD). Data were analyzed using SPSS 20.0 statistical software. One-way ANOVA was used to compare the differences in the lignin degradation rates and the LiP activity results. All graphs in results were produced using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA, 1989).

3. Results and Discussion

3.1. Screening and Identification of Ligninolytic Strains

In this study, three ligninolytic strains were classified as *Enterobacter* BS0669, *Enterobacter* BS1957, and *Enterobacter hormaechei* KA3 by 16S rRNA sequencing and NCBI comparison. For determining the highest lignin-degrading ability, the lignin degradation ability of these strains was analyzed and compared. Results showed that the strain *Enterobacter hormaechei* KA3 had the highest lignin-degrading rate followed by *Enterobacter* BS0669 and *Enterobacter* BS1957 after seven days at 37 °C (shown in Figure S1, Supplementary Materials). Therefore, *Enterobacter hormaechei* KA3 was selected for subsequent AD experiments.

3.2. Lignin-Degrading Capability of Enterobacter Hormaechei KA3

3.2.1. Lignin-Degrading Rate

According to Section 3.1, the highest lignin-degrading rate of 32.05% was obtained for CS treatment using strain KA3 compared to the two other strains (shown in Figure S1, Supplementary Materials). In previous studies, many lignin-degrading strains have been reported. For example, Yang et al. found that the degradation rate was only 8.25% after seven days of treatment with the screened *Burkholderia* sp. H1 for wheat straw, and the rate rose to 16.74% after 15 days of treatment [24]. The lignin degradation rate was 17.21% after five days of treatment at 60 °C for *Geobacillus thermodenitrificans* Y7 isolated by Ma et al. [25]. In addition, the same genus of the lignin-degrading strain *Enterobacter hormaechei* PY12 used in this study was also isolated from termite gut [11]. However, the lignin-degrading rate was still lower compared with the degradation rate of strain KA3.

3.2.2. Enzyme Activity

The enzymatic reaction conditions (pH, temperature, and reaction time) of strain KA3 were optimized. As shown in Figure S1 (Supplementary Materials), the activity of lignin peroxidase (LiP) and laccase (Lac) of strain KA3 in liquid culture for a different number of days was determined. The results indicated that LiP enzyme activity (585.2 U/L) was highest on the fifth day of incubation while Lac activity was consistently close to zero. Therefore, strain KA3 degrades lignin by the LiP degrading pathway. In addition, the optimal and temperature for LiP activity were 5.0 and 37 °C, respectively.

At present, most studies have assessed the lignin-degrading ability of microorganisms by measuring the activity of lignin-decomposing enzymes secreted by organisms, including Lac, LiP, and manganese peroxidase (MnP). The LiP activity of *Enterobacter hormaechei* Py12 and *Bacillus licheniformis* MX5 screened from termite intestines was 278 U/L and 256 U/L, respectively [11]. The LiP enzyme activities of L3, L9, and L1 of *Bacillus* screened from jute, decayed material of fallen trees, and soil were 571.2 ± 2.2 (U L^{-1} min^{-1}), 615.6 ± 2.6 (U L^{-1} min^{-1}), and 558.7 ± 2.1 (U L^{-1} min^{-1}), respectively [26]. In this study, the LiP activity of strain KA3 was 585.2 U/L after seven days treatment with CS. This value is similar to the enzyme activities mentioned above. This indicates that the strain KA3 can effectively decompose the lignin in crop straw, which is beneficial for the practical application of biogas conversion of crop straw in the later stage.

3.2.3. Intuitive Analysis of Strain KA3 with CS Treatment

To further verify the lignin-degrading ability of strain KA3, the surface structure of CS was observed after treatment using SEM (shown in Figure S2). SEM in S2 clearly showed that strain KA3 caused sufficient damage to the CS in the treated group, whereas the surface of the CS remained smooth in the untreated group. This is consistent with previous research reports that the surface of treated corn stalks is no longer smooth and has varying degrees of damage [27]. The SEM images of CS treated with the fungus didymosphaeria sp. cmu-196 were presented in the study by Arredondo Santoyo et al., but the difference is that the structural modification of vascular tissue was exhibited [8]. The above results illustrated that the screened *Enterobacter hormaechei* KA3 had significant lignin-degradation ability.

3.3. Effect of Strain KA3 on AD of CS

3.3.1. Biogas/Methane Production

The biogas/methane yields of TG and CK after 35 days of anaerobic digestion are shown in Figure 1. Better process stability was achieved in TG considering the daily biogas yield compared with CK. The highest daily biogas yield of TG (5.11 L/kg-VS) and CK (4.13 L/kg-VS) appeared after digestion for 14 days and 22 days (Figure 1a). It indicated that strain KA3 could decrease the digestion period, and the highest daily methane yields of TG (2.81 L/kg-VS) and CK (2.35 L/kg-VS) were also obtained (Figure 1c). These values showed no significant difference. Compared with cumulative biogas yield (Figure 1b), the value of TG (59.19 L/kg-VS) was significantly increased by about 20% compared with that of the CK (47.48 L/kg-VS, $p < 0.05$). In addition, in terms of cumulative methane yield (Figure 1d), TG (14.76 L/kg-VS) increased by about 31% compared to CK (10.16 L/kg-VS). Therefore, the addition of strain KA3 increased the degradation rate of lignin and the conversion rate of substrates to biogas.

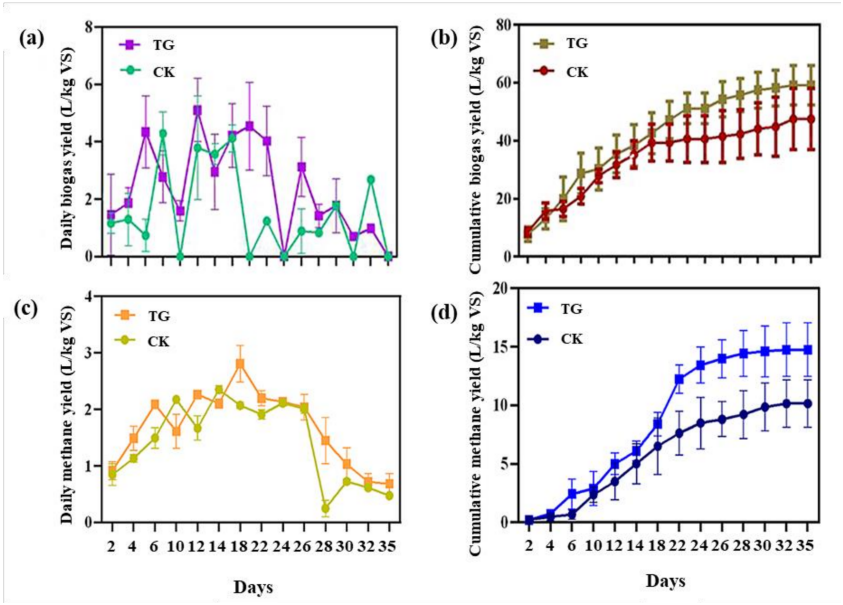

Figure 1. Biogas/methane production (L/kg-VS) of AD. (**a**) Daily biogas yield, (**b**) Cumulative biogas yield, (**c**) Daily methane yield, (**d**) Cumulative methane yield. Results are presented as mean values and SD for three replicates.

However, in the study of Rahimi et al., the addition of strain TAV5 in the digestion system increased the methane production of yard waste, mixed waste, and wood by 34%, 49%, and 297%, respectively [28]. When *'cellulolytic archaea' norank_c_Bathyarchaeia* was enriched and used in a bioaugmentation experiment, the daily methane yield rate was significantly increased by 24.3% [29]. According to Shah et al., the methane yield of wheat straw treated with lignin-degrading bacillus was 261.4% higher than that of untreated wheat straw [30]. Pretreatment of lignocellulosic substrate with cattle rumen fluid could increase methane production by 36.18% [31]. The increased values of this study are consistent with these values. However, in contrast to these reported studies, the biogas/methane production was lower in our experiment. The reasons may be that the total reaction system of the digestion apparatus in this study is much less, and the contact area of the substrate is not sufficient, thus limiting the degradation efficiency of the substrate.

3.3.2. Substrate Conversion Rate

Changes of VFA concentration and the removal rates of COD, TS, and vs. were used to evaluate the substrate conversion rate. All values are shown in Figure 2. VFA is the intermediate in the acetylogenic and acidogenic phases of AD, and methanogens mainly utilize VFA to produce methane. Therefore, the change in VFA concentration can indirectly reflect the overall performance and stability of the AD system process shown in Figure 2a. It was obvious that as the digestion process progresses, the VFA concentration increased firstly, then decreased. In the early phase (2–17 days in this study), substrates are degraded and most products are used to generate VFA. In the anaphase (18–35 days in this study), VFAs were constantly consumed to produce methane. This could be explained by the acetic acid concentration (Figure 2b), which had the same variation trend as VFA. Acetic acid is the main precursor substrate for methane production, and it was lower in TG than in CK. Therefore, VFA and acetic acid gradually decreased, and more biogas/methane yield was found in TG. The highest concentrations were obtained in the medium stage (18D) for both groups: 2427 mg/L (TG) and 2314 mg/L (CK) with no significant difference. These results are consistent with the changes in digestion parameters when lignocellulose-degrading bacteria isolated from termite digestive tracts were added to rumen fluid [32]. However, the process of TG had better stability during the whole digestion period. The reason might

be that the added strain KA3 had no strong synergistic effect with digestion acid-producing bacteria.

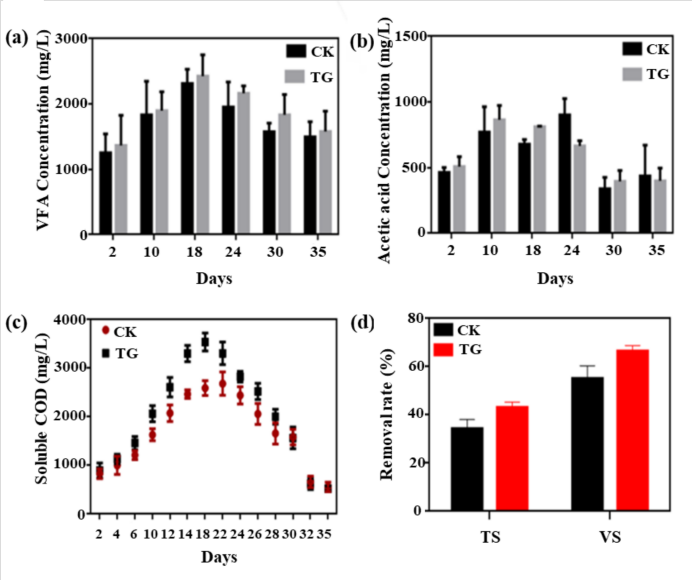

Figure 2. The substrate utilization efficiency for AD. Variation trends of (**a**) VFA, (**b**) acetic acid, and (**c**) COD concentrations (mg/L) at different digestion times. (**d**) Removal rates (%) of TS and vs. after digestion. Results are presented as mean values and standard deviations for three replicates.

The removal rates of COD, TS, and vs. also can reflect the decomposition and conversion of substrates by microorganisms. These indicators can reveal the utilization rate of substrates and the digestion efficiency. As shown in Figure 2c, the conversion of substrate to biogas was similar to that for VFA and acetic acid. The COD contents of TG and CK reached the peak values on 18D and 22 days (22D), which were 3540 mg/L and 2686 mg/L. These values increased by 74.6% and 71.03% over the initial concentration, respectively. At the end of the digestion period, they decreased by 41.6% and 32.73%, respectively, compared with the initial concentrations. This result is consistent with the results shown above for biogas production. The removal rate of TS and vs. after fermentation in each group is shown in Figure 2d. In terms of TS, the removal rate in TG was 43.11%, while that in CK was 34.33%, and the vs. removal rate was basically consistent with the results of TS; it was 66.59% in TG and 55.16% in CK. Higher TS and vs. removal rates were achieved in TG than in CK, attributed to the higher substrate conversion to biogas/methane (higher VFA and COD concentration and biogas/methane production).

In previous studies, different degrees of vs. reduction rates (16% to 31%) were obtained when anaerobic digestion was performed with the CM from different farms, while vs. reduction rates could be increased to 59% when co-digested with crop straw. COD removal rates ranged from 30% to 35% [33]. *Pleurotus ostreatus* was confirmed to have a higher vs. removal rate of 32%, and the vs. removal rate of *Schyzophyllum commune* was 11% [34] when treated with crop straw. However, these values are still lower than that obtained in this study. Therefore, using strain KA3 to treat CS could effectively improve the conversion rate of the substrate to biogas in the AD system, making the substrate utilization more efficient.

3.4. Effects of Strain KA3 on the Microbial Community in the AD System

3.4.1. Archaeal Community Characteristics

The reading length ranged from 200 bp to 450 bp (archaea and bacteria). And the analyzed reads were detected based on 97% sequence identity. The α-diversity of archaea and bacteria was showed in Table 1. The Shannon index was higher in TG (ranged from 2.17 to 2.30) than in CK (ranged from 1.69 to 1.97) and higher at 18D than at 7D. The Simpson index, ACE index and Chao1 index showed the same variation features of α-diversity.

In addition, the β-diversity analysis of archaea exhibited in Figure 3a illustrated that a significant difference was found between TG and CK at 7D and 18D. Thus, β-diversity showed a significant difference. Various studies have reported that microbial community description indexes such as the diversity and evenness index are directly related to changes in anaerobic digestion function [35–37]. These NMDS plots were relatively dispersed, which clearly indicates changes in the community structure at different digestion times and groups.

Table 1. α-diversity analysis index of different communities.

Category	Samples	Shannon	Simpson	ACE	Chao1
Archaea	CK-7D	1.69	0.37	75.51	73.38
	CK-18D	1.97	0.24	69.22	73.33
	TG-7D	2.17	0.22	131.78	96.75
	TG-18D	2.30	0.16	108.76	94.67
Bacteria	CK-7D	3.91	0.06	667.44	654.36
	CK-18D	3.76	0.06	693.33	701.43
	TG-7D	4.00	0.05	706.00	697.75
	TG-18D	4.20	0.03	713.06	705.25

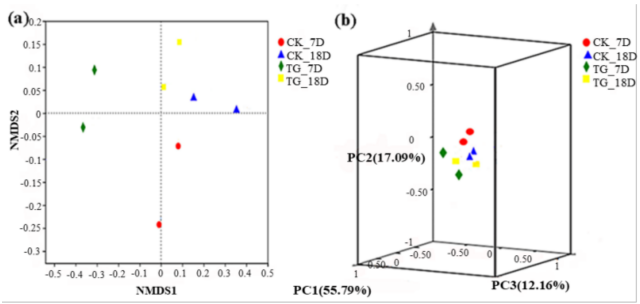

Figure 3. β-diversity analyses of the archaeal community (**a**) and bacterial community (**b**). The red spheres and blue triangles represent the 7D and 18D of digestion in CK, and the green diamonds and yellow squares represent the 7D and 18D of digestion in GT.

The archaeal community composition and structure are shown in Figure 4. As the AD process prolonged, the relative abundance changed for different taxa. In terms of the phylum level, *Halobacterota*, *Euryarchaeota*, and *Thermoplasmatota* were the dominant taxation both in TG and CK. It is evident that from the 7th day to the 18th day, the abundance of *Halobacterota* in TG increased from 91.95% to 96.1%, while in CK, the abundance increased from 78.04% to 90.49%, which showed no significant difference ($p > 0.05$). At the genus level, the composition of dominant taxa in TG changed significantly at different times. The dominant taxa were *Methanosaeta*, *Methanomoicrobiales*, and *Methanoculleus*, compared with *Methanosaeta* and *Methanosarcina* with a significant increase in *Methanosarcina* from 5.01% at 7D to 41.21% at 18D and a decrease in *Methanosaeta* from 45.36% to 39.82%, *Methanomicrobiales* (from 20.34% to 3.96%), and *Methanoculleus* (from 17.87% to 6.49%). In the AD process, *Methanosarcina* and *Methanosaeta* can directly utilize acetate formed by acetogenic bacteria [38]. *Methanosaeta* is an obligate acetolytic methanogen and has a higher competitiveness of acetate compared with *Methanosarcina* [39]. For CK, the dominant taxa were *Methanosarcina* and *Methanosaeta*, but the abundance of *Methanosarcina* increased at 18D. Thus, for TG, methane generation mainly contributed to the acetic acid-pathway. Furthermore, the hydrogenotrophic methanogen (e.g., *methanocella*) can maintain low concentrations of H_2 in the system, allowing syntrophics to produce high concentrations of acetate [40]. The results of this study illustrated that mainly hydrogenotrophic methanogenesis was performed and the concentration of acetate was low during the early period of fermentation, probably due to the hydrolyzing bacteria as the main flora, which inhibited the abundance of acidogenic bacteria. The abundance of *Methanosarcina* increased sharply

due to the high concentration of acetate in the middle digestion stage, and the degradation of the substrate was mainly carried out in the early stage of digestion.

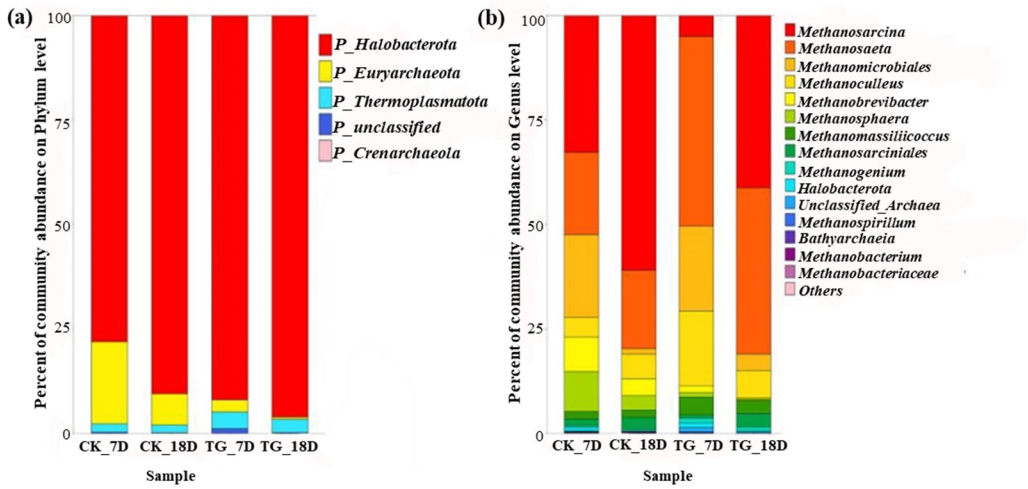

Figure 4. Archaeal community composition and structure at (**a**) the phylum level and (**b**) genus level. Statistical analysis was performed by Student's *t* test. Note: $p < 0.05$. CK-7D and TG-7D were the samples of the control group and the treatment group on the 7th day. CK-18D and TG-18D were the samples of the control group and the treatment group on the 18th day.

3.4.2. Bacterial Community Characteristics

Considering the diversity, the α-diversity showed the same variation tendency as archaea. For Shannon index, it was higher in TG (ranged from 4.00 to 4.20) than in CK, but it decreased in CK (ranged from 3.91 to 3.76) at 7D to 18D (Table 1). This is the reason why strain KA3 promoted the decomposition of substrate and thus improved the hydrolytic bacterial activity. The β-diversity of bacteria was similar to that of archaea (Figure 3b), which showed a significant difference between TG and CK.

The AD process not only involves methanogenic archaea, but also some hydrolytic and acidogenic bacteria that participate in the hydrolysis stage and acidogenic stage. At the phylum level and genus level, the dominant taxa showed no significant difference ($p > 0.05$). At the phylum level, *Firmicutes*, *Bacteroidota*, *Actinobacteriota*, and *Proteobacteria* had higher abundance both in TG and CK (Figure 5a) at different digestion times. At the genus level (Figure 5b), the dominant genera of TG and CK were also similar: *Solibacillus*, *Clostridium*, and *Sporosarcina*. *Ruminofilibacter* and *Bacillus* also had a relative high abundance. *Solibacillus* can secrete different enzymes for substrate degradation, and *Clostridium* was an important microorganism in degrading proteins and polysaccharides in previous studies [41,42]. Therefore, they were widely present in CK and TG. Many studies have reported that *Ruminofilibacter* can effectively degrade lignin substrates [43–45]. *Bacillus* was confirmed as a good species that can utilize carbohydrates with high efficiency [44]. The increased abundance of *Bacillus* and *Ruminofilibacter* in TG might be due to the synergistic hydrolysis of bacteria with KA3 to degrade substrates together. The carbohydrates can be converted into soluble sugar for biogas production in the later stage to increase methane yield.

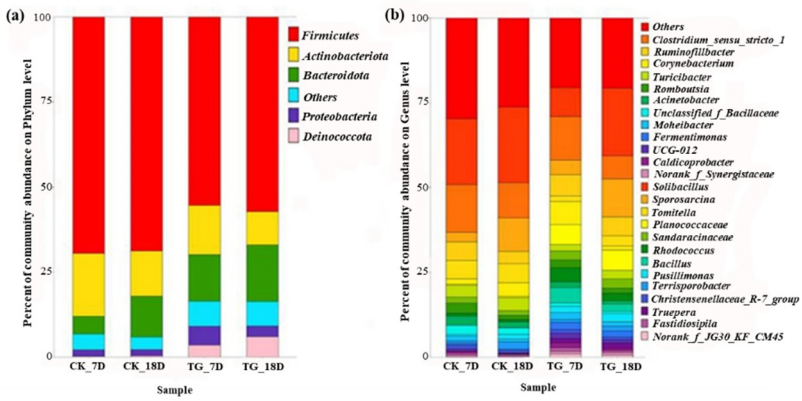

Figure 5. Bacterial community composition and structure at (**a**) the phylum level and (**b**) genus level. Statistical analysis was performed by Student's *t* test. Note: $p < 0.05$. CK-7D and TG-7D were the samples of the control group and the treatment group on the 7th day. CK-18D and TG-18D were the samples of the control group and the treatment group on the 18th day.

3.5. Practical Implementation

Agricultural solid organic waste (ASOW) is one of the main factors affecting sustainable development in China. Among ASOWs, CS is considered an ideal candidate for clean energy production [46]. However, improper disposal of corn stalks such as abandonment or burning in the field not only wastes resources, but also causes serious safety and environmental problems [3]. Therefore, effective lignin degradation is the key to improve the efficient utilization of lignocellulosic biomass energy. The lignin-degrading strain KA3 screened from the gut microbes of *Hypomeces squamosus* Fabricius can effectively destroy the lignin structure on the surface of CS, so as to improve the degradation efficiency of CS. On the one hand, the degraded CS could be applied in anaerobic fermentation to produce biogas to realize the recycling of biomass and reduce environmental pollution to protect the environment. On the other hand, the diverse phenolic intermediates of lignin degradation can be used to produce high value-added chemicals with low molecular weight to improve the economic benefits of lignin industrialization [47]. However, the specific lignin degradation mechanism and metabolic pathway of this strain are still unclear, and further studies are needed for its industrial production application.

4. Conclusions

In this study, three bacterial strains for lignin degradation were screened and identified from the gut microbiomes of lignin-feeding *Hypomeces squamosus* Fabricius collected from the Xiama forest farm in Tianzhu, China. In addition, the isolated lignin-degrading bacteria were successfully used for the biomethane conversion of lignin. The lignin-degradation ability of strains was analyzed, and the highest degradation for CS-*Enterobacter hormaechei* KA3 (32.05%) was obtained. To evaluate the application potential strain of KA3 in biogas conversion of crop straw, it was used in the AD system of CS, and the effects were investigated. Results showed that strain KA3 degraded lignin by secreting LiP with a high activity of 585.2 U/L. Furthermore, the highest accumulative biogas yield and methane yield were obtained for KA3 inoculation, which increased by 20% and 31% compared to CK. Higher VFA and acetic acid concentrations and removal rates of COD, TS, and vs. were also found. Therefore, inoculation of strain KA3 in the AD system improved the decomposition of lignin and further enhanced the substrate conversion rate and biogas/methane production. Moreover, microbial community diversity increased as digestion time prolonged in TG. *Methanosate* was the dominant genus of methanogens in TG, compared with *Methanosarcina* in CK. For bacteria, dominant taxa were similar for all groups, i.e., *Solibacillus* and *Clostridium*. Therefore, strain KA3 improved the biogas/methane production efficiency by improving the precursor matter and acetic acid pathway for biogas generation. Lignin is the second largest biomass carbon source in nature and has great application

potential. However, its efficient degradation is a bottleneck, and the identified resources of lignin-degrading bacteria are limited. This study could provide a new microbial resource and practical application base for lignin degradation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/en14112990/s1, Figure S1: The lignin degradation ability of the strain was screened; Figure S2: SEM characterization of surface changes after treatment of corn straw with *Enterobacter hormaechei* KA3; Table S1: Characteristics of substrates and inoculum.

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Abbreviations

AD	anaerobic digestion
CS	corn straw
LB	Luria–Bertaini
SW	saline water
SEM	scanning electron microscope
CM	cow manure
TG	treated group
CK	control group
VFA	volatile fatty acid
COD	chemical oxygen demand
7D	digested for seven days
18D	digested for 18 days
22D	digested for 22 days
TS	total solid
VS	volatile solid
TOC	total organic carbon
SD	standard deviation
LiP	lignin peroxidase
MnP	manganese peroxidase
Lac	laccase
ASOWs	agricultural solid organic waste

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