Biomethane production from sugar beet pulp under cocultivation with *Clostridium cellulovorans* and methanogens

Hisao Tomita¹, Fumiyoshi Okazaki¹,²,³ and Yutaka Tamaru¹,²,³*

**Abstract**

This study was demonstrated with a coculture fermentation system using sugar beet pulp (SBP) as a carbon source combining the cellulose-degrading bacterium *Clostridium cellulovorans* with microbial flora of methane production (MFMP) for the direct conversion of cellulosic biomass to methane (CH₄). The MFMP was taken from a commercial methane fermentation plant and extremely complicated. Therefore, the MFMP was analyzed by a next-generation sequencing system and the microbiome was identified and classified based on several computer programs. As a result, *Methanosarcina mazei* (1.34% of total counts) and the other methanogens were found in the MFMP. Interestingly, the simultaneous utilization of hydrogen (H₂) and carbon dioxide (CO₂) for methanogenesis was observed in the coculture with Consortium of *C. cellulovorans* with the MFMP (CCeM) including *M. mazei*. Furthermore, the CCeM degraded 87.3% of SBP without any pretreatment and produced 34.0 L of CH₄ per 1 kg of dry weight of SBP. Thus, a gas metabolic shift in the fermentation pattern of *C. cellulovorans* was observed in the CCeM coculture. These results indicated that degradation of agricultural wastes was able to be carried out simultaneously with CH₄ production by *C. cellulovorans* and the MFMP.

**Keywords:** Methanogenesis, Cellulosic biomass degradation, Coculture, Gas metabolism

**Introduction**

Although first-generation biofuels are made from cones and sugarcanes to mainly produce bioethanol by using yeast, they are crops and foods without doubt that led accordingly to the food problem. For certain reasons, second-generation biofuels are produced from non-edible biomass such as agricultural wastes and cellulosic substrates (Naik et al. 2010; Schenk et al. 2008). Furthermore, the investigation of third-generation biofuels made from algae has been started (Alam et al. 2015). Thus, considering competition with food supply in response to an increase in global population, it is desirable to overcome first-generation biofuels, proceeding to next-generation biofuels.

Cellulose is comprised of a linear chain of d-glucose monomers and has strong crystalline (Brethauer and Studer 2015). Moreover, cellulosic biomass is composed of cellulose, hemicellulose and lignin, and has rigid and complex structures (Gray et al. 2006). Hemicellulose is heteropolymer such as xylan, glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan. In addition, lignin, phenol compounds, are assembled with cellulose and hemicellulose. Thus, since rigid and complex structures are constructed in cellulosic biomass, it is very difficult to degrade them enzymatically.

About 20% of the world’s sugars is supplied by a root of a sugar beet (*Beta vulgaris* L.), which are cultivated all over the world mostly in Europe, North America and Russia (FAO—agribusiness handbook 2013). Sugar beet pulp is a by-product of the production of sugar from the sugar beet. The extraction of sugar starts with the cleaning of the sugar beet delivered to the factory, after which the sugar beet is sliced up into small strips (pulp) and then mashed by heating with water to a temperature of...
approximately 70 °C to dissolve sugars from the pulp. Furthermore, the sugar water and the pulp are separated in an extraction tower. Thus, since sugar beet pulp (SBP) is the residue and non-edible biomass, it was the subject of research into a raw material of second-generation biofuels (Bellido et al. 2015; Zheng et al. 2013). Furthermore, SBP is mainly composed of cellulose, arabinan and pectin, has less lignin. Therefore, SBP is a suitable raw material for second-generation biofuels, because a pretreatment process is not necessary to remove lignin (Table 1).

Some species of Clostridia are known to have ability to degrade cellulosic biomass efficiently using a multi-enzyme complex called the cellulosome and secreted non-cellulosomal enzymes (Doi and Kosugi 2004). Among those species, we have been studying on *Clostridium cellulovorans*, which is a mesophilic and anaerobic cellulytic bacterium (Sleat et al. 1984). *C. cellulovorans* utilizes not only cellulose but also hemicelluloses consisting of xylose, fructose, galactose, and mannose (Koukiekolo et al. 2005; Beukes et al. 2008; Dredge et al. 2011). Whole-genome sequencing of *C. cellulovorans* and the exoproteome profiles revealed 57 cellulosomal protein-encoding genes and 168 secreted-carbohydrase-encoding genes (Tamaru et al. 2010; Matsui et al. 2013). Furthermore, since high ability of degradation on plant cell walls has so far been reported (Tamaru et al. 2002), researches have continued to study on degradation mechanism for cellulosic biomass such as rice straw by *C. cellulovorans* (Nakajima et al. 2017).

**Materials and methods**

**Materials**

SBP was obtained from a sugar factory in Hokkaido, Japan. It was dried up, milled and sieved through 80 mesh. The substrate concentration of SBP and Avicel (Sigma, MO, USA) was 0.5% (w/v) of dry weight.

**Microorganism and culture condition**

The medium was partially modified by *Clostridium cellulovorans* medium (Sleat et al. 1984). One liter medium containing 4 g of yeast extract, 1 mg of Resazurin salt, 1 g of l-cysteine-HCl, 5 g of NaHCO₃, 0.45 g of K₂HPO₄, 0.45 g of KH₂PO₄, 0.3675 g of NH₄Cl, 0.9 g of NaCl, 0.1575 g of MgCl₂·6H₂O, 0.12 g of CaCl₂·2H₂O, 0.85 mg of MnCl₂·4H₂O, 0.942 mg of CoCl₂·6H₂O, 5.2 mg of Na₂EDTA, 1.5 mg of FeCl₂·4H₂O, 0.07 mg of ZnCl₂, 0.1 mg of H₃BO₃, 0.017 mg of CuCl₂·2H₂O, 0.024 mg of NiCl₂·6H₂O, 0.036 mg of Na₂MoO₄·2H₂O, 6.6 mg of FeSO₄-7H₂O, and 0.1 g of p-aminobenzoic acid and was adjusted to pH7. *C. cellulovorans* 743B (ATCC 35296) was used and anaerobically cultivated in 0.5% (w/v) cellulose (Sigma, MO, USA) at 37 °C for 19 h stationary. The MFMP was obtained from methane fermentation

| Component | Weigh (g) per dry matter (100 g) |
|-----------|---------------------------------|
|           | Hadden et al. (1986) | Zheng et al. (2013) |
| Ash       | 3.42 g/100 g | 2.51 g/100 g |
| Proteins  | 11.42 g/100 g | 11.42 g/100 g |
| Lipids    | 1.63 g/100 g | – |
| Sugars*   | 5.2 g/100 g | – |
| Starch    | 0.99 g/100 g | – |
| Lignin    | 2.38 g/100 g | 1.16 g/100 g |
| Glucan    | 17.34 g/100 g | 22.7 g/100 g |
| Xylan     | 1.36 g/100 g | 5.14 g/100 g |
| Galactan  | 4.88 g/100 g | 5.92 g/100 g |
| Arabinan  | 16.83 g/100 g | 23.73 g/100 g |
| Mannan    | 1.58 g/100 g | 1.85 g/100 g |
| Pectin    | 21.15 g/100 g | 22.84 g/100 g |
| Others    | – | 2.73 g/100 g |

* Total value of rest of fructose, glucose, sucrose and fructan
b Conversion of values to polysaccharides in the paper

Methane fermentation is conventional-generation biofuels, and many researches have been reported in a wide range of study fields (Guo et al. 2015). Since methane production is carried out by the complex microbial flora included methanogens, it was formerly difficult to grasp the whole of the microbial flora. However, it has now become possible to analyze the whole aspect of the microbiome characteristics using the next-generation sequencing system (Spang et al. 2017). It has been reported on coculturing with *C. cellulovorans* and one of the famous methanogens such as *Methanosarcina* spp. (Lu et al. 2017). Since *C. cellulovorans* and methanogens were able to grow anaerobically under mesophilic condition, it was possible to cultivate both of them in a single tank and simultaneously with both the degradation of cellulosic biomass and production of methane (CH₄).

In the present study, we investigated a process for producing CH₄ and hydrogen (H₂) via the coculture of *C. cellulovorans* with microbial flora of methane production (MFMP) that called the Consortium of *C. cellulovorans* with MFMP (CCeM) with carbon sources such as SBP and Avicel. First, we analyzed 16 s rRNA sequences in the MFMP by using a next-generation sequencer. Based on the result of identification of the MFMP microbiome, both *C. cellulovorans* and the MFMP monocultures and the CCeM coculture were carried out to evaluate concentrations of sugars, organic acids, and biogas (H₂ and CH₄) yield after cultivation.
digested liquid on January, 2017 at Gifu in Japan. The MFMP was anaerobically cultivated in Clostridium cellulovorans medium with 0.5% (w/v) glucose (Wako) and 0.25% (w/v) cellobiose at 37 °C for 19 h stationary.

16S rRNA sequencing
Samples were crashed by Shake Master Neo (bms, Tokyo, Japan) and DNA was extracted by Fast DNA spin kit (MP Bio, CA, USA). MiSeq (Illumina, CA, USA) was used for sequencing under the condition of $2 \times 300$ bp. Qiime as an analyzing software and Greengene as a database were used, and OTU was decided except chimeric genes.

Measurement of total sugar and reducing sugar concentration
Total sugar concentration was measured by Phenol–sulfuric acid method. Reducing sugar was measured by DNS method, as d-glucose equivalents.

Data deposition
The sequences reported in this paper has been deposited in the DDBJ database (accession no. DRR160954).

Gas concentration
Produced gas after the cultivation was recovered by downward displacement of water, the total gas amount was measured by a syringe (Terumo, Tokyo, Japan). The concentration of CH$_4$, H$_2$ and CO$_2$ was measured by a gas chromatograph GC-8A (Shimadzu, Kyoto, Japan) with TCD detector and a column SINCARBON ST (6 m, inner diameter. 3 mm; Shinwa, Kyoto, Japan). The column temperature was at 200 °C. Argon was a carrier gas and set at a flow rate of 50 mL/min. Injection volume of each sample was 5 mL.

Organic acid concentration
The concentration of organic acids was measured by high-performance liquid chromatography (HPLC) CBM-20A, LC-20AD, CTO-20AC, SPD-20A and DGU-20A$_3$ (Shimadzu, Kyoto, Japan) with UV detector and a column KC-811 (300 mm x 2, inner diameter. 8 mm; Showa Denko, Tokyo, Japan). The column temperature was at 60 °C. The method of BTB Post-column was used. Eluent was 2 mM perchloric acid, and the flow rate was 1.0 mL/min. Reagent was 0.2 mM BTB and 15 mM disodiumhydrogenphosphate, and the flow rate was 1.2 mL/min at the wavelength of 445 nm. Injection volume of each sample was 20 μL.

Cell growth
Cell growth was measured by Lumitester PD-20, LuciPac Pen and ATP eliminating enzyme (Kikkoman Biochemifa, Tokyo, Japan). It is known that integrated intracellular ATP concentration correlates with cell growth (Miyake et al. 2016). Cell growth was estimated by measuring ATP concentration of 0.1 mL of cell culture according to the manufacturer’s instruction and was expresses by Relative Light Unit (RLU) value.

Statistics
The data were analyzed for statistical significances using Welch’s t test. Difference was assessed with two-side test with an α level of 0.05.

Results
Degradation of SBP and Avicel with C. cellulovorans
Anaerobic batch cultivations of C. cellulovorans were carried out in a 40-mL medium containing 0.5% (w/v) of SBP at 37 °C without shaking. After cultivation with SBP, the volume became less than half of the negative control (Fig. 1). Next, Avicel was used for a reference of cellulose degradation with C. cellulovorans. According to measured cell growth on the precultures, the inoculation volume with a C. cellulovorans monoculture was decided. As a result, the initial RLU value of the monoculture closely reached to 1000, whereas the RLU value of the C. cellulovorans preculture with 0.5% (w/v) cellobiose was 20,257. Therefore, the inoculation volume was eventually decided to 2 mL for 40-mL monoculture which was about 21 times dilution, so that the initial RLU value of the C. cellulovorans monoculture was 964. The concentration of total sugar, reducing sugar and organic acids, cell growth and gas production were measured for 11-days cultivations, respectively. C. cellulovorans degraded 87.3% SBP and 86.3% Avicel, respectively, without any pretreatment (Fig. 2a). Interestingly, the profiles of cell growth with C. cellulovorans on both SBP and Avicel cultures were completely different (Fig. 2b). The maximum cell growth
in the Avicel culture was 5-days after inoculation, while that in the SBP culture was 1-day after inoculation. On the other hand, whereas the concentration of butyric acid increased rapidly on the Avicel culture after 2-days inoculation, there was no increase of butyric acid on the SBP culture (Fig. 2c). It was suggested that a metabolic pathway in C. cellulovorans might be different between the SBP and Avicel cultures. According to the concentrations of reducing sugar in the SBP and Avicel cultures, they seemed very similar (Fig. 2d). However, H₂ productions were 28.6 L per 1 kg of dried SBP and 132 L per 1 kg of Avicel, respectively (Fig. 2e). Therefore, the composition of the reducing sugar in the SBP culture seems reasonable to produce 28.6 L of H₂ whose concentration was close to 22% of 132 L of H₂ in the Avicel culture. Thus, it indicated that C. cellulovorans degraded cellulosic biomass to produce H₂ which should be a raw material of CH₄ by the CO₂ reduction pathway in methanogens.

All-inclusive analysis of microbial flora included methanogens
Based on the 16S rRNA sequencing, a total of 2359 OUT IDs has read counts from analyzing 24,105 OUT IDs. Eventually, 17 classes and their species were identified among them (Table 2). In fact, whereas Clostridium butyricum was identified as the same species of C. cellulovorans, Methanosarcina mazei (1.34%) was found among methanogens. Furthermore, other methanogens such as Methanotaetaceae, Methanoseta, and Methanospirillaceae were also identified. More interestingly, the genus Methanoseta, which utilizes only acetic acid, was a large portion of ratio next to Methanosarcina (Table 3). Dominant families were identified and belonging to Syntrophomonadaceae (11.37%), Marinilabiacae (5.59%), Clostridiaceae (4.91%), and Spirochetaeceae (4.52%) (Fig. 3).

Precultivation of C. cellulovorans and MFMP
The inoculation volume to the MFMP monoculture was decided as same as the C. cellulovorans monoculture, so that the initial RLU values of the MFMP monoculture closely reached to 1000. The RLU value of the MFMP preculture with 0.5% (w/v) glucose and 0.25% (w/v) cellulbiose was 14,812. Therefore, the inoculation volume was decided to 3 mL for 40-mL monoculture, so that the initial RLU value of the MFMP monoculture was 1036. 2 mL of the C. cellulovorans preculture and 3 mL of the MFMP preculture, respectively, were inoculated in the CCeM culture, in order that the concentration of cell growth against the substrate became same as monocultures.

Methanogenesis and SBP utilization
Anaerobic batch cultivations of the CCeM and MFMP cultures were carried out in a 40-mL medium containing 0.5% (w/v) of sugar beet pulp at 37 °C without shaking. The total sugar of the MFMP culture was hardly decreased. However, surprisingly, the total sugar of the CCeM culture decreased 86.0% that was not significantly deference compared with that of C. cellulovorans monoculture (Fig. 4a). Furthermore, cell growth of the CCeM culture was higher than that of the MFMP culture during 2–6 days cultivation as with the RLU profile of the C. cellulovorans monoculture (Fig. 4b), and the butyric acid concentration in the CCeM culture was higher than that in the MFMP culture (Fig. 4c). Whereas pH in the C. cellulovorans culture with Avicel after 11-days cultivation was 6.4 due to the high concentration of butyric acid, pH in the CCeM culture with SBP was 6.57 (Fig. 4e). On the other hand, the reducing sugar concentration decreased from the initial value in the CCeM and MFMP cultures, and CO₂ production in both cultures were two times higher than that in the C. cellulovorans monoculture (Fig. 4f). It suggested that various microbes in the MFMP consumed the reducing sugar and produced CO₂ in the CCeM and MFMP cultures. Thus, it was demonstrated that C. cellulovorans was able to coexist with methanogens and various other microbes to degrade SBP, while the degradation performance of C. cellulovorans was maintained. For biogas production, 34.0 L/kg of CH₄ and 110 L/kg of CO₂ were measured in the CCeM culture, respectively. On the other hand, 48.2 L/kg of CH₄ and 105 L/kg of CO₂ in the MFMP culture were done, respectively. It was also revealed that MFMP was able to produce CH₄ coexisting C. cellulovorans. More interestingly, H₂ was not accumulated in both cultures, and the final volume of H₂ was less than that in negative control, although 28.6 L/kg H₂ was produced in the C.
cellulovorans monaculture (Fig. 4d). These results suggested that *M. mazei* generated CH4 from H2 and CO2 by the CO2 reduction pathway.

**Discussion**

The biomethanation process is not a single process. Three anaerobic microbes such as fermentative microbes, acetogenic microbes and methanogens mainly participate in the methanation (Hattori 2008; Thauer et al. 2008; Garcia et al. 2000). In fact, methanogens required acetate, H2 and CO2, which are precursors for methanogenesis, to metabolite CH4 by two major pathways such as the aceto-clastic pathway and the CO2 reduction pathway (Deppenmeier et al. 1996). Fermentative and acetogenic microbes degrade organic matters and supply the precursors to methanogens. A physiological and molecular investigation of two artificially constructed co-cultures with *C. cellulovorans–M. barkeri* utilizing cellulose as the sole carbon source has been reported (Lu et al. 2017). In this study, whereas *C. cellulovorans* was able to grow on the medium containing 0.5% cellobiose, some bacteria can never utilize...
it. In fact, after cultivation of \textit{C. cellulovorans} with the Avicel medium, main hydrolyzed products were cellobiose in the supernatant, suggesting that only glucose might be used for methane production by MFMP. On the other hand, since however \textit{C. cellulovorans} degraded SBP to produce a variety of saccharides which could be utilized by various microbes in MFMP. Therefore, SBP would be a great benefit to reduce the cost of drying and transporting SBP in sugar factories. Exoproteome analysis of \textit{C. cellulovorans} under the cultivation with several substrates such as bagasse, corn germ, and rice straw revealed that 18 of the proteins were specifically produced during degradation of types of natural soft biomass (Esaka et al. 2015). More interestingly, in comparison of the cocultures between \textit{C. cellulovorans}–\textit{M. berkeri} and \textit{C. cellulovorans}–\textit{M. mazei}, the pattern of gene expression on a cellulose encoding \textit{Clo}cel\_0905 was completely different from the combination between \textit{M. berkeri} and \textit{M. mazei} (Lu et al. 2017). This result indicated that it might have another possibility of cellulose degradation manners via microbial interactions. In this study, the concentration of butyric acid in SBP culture did not increase much, although that of acetic acid immediately increased for 1-day cultivation (Fig. 2b, c), suggesting that \textit{C. cellulovorans} grew and produced butyric acid and the starting point of its growth was delayed until cellulosome and non-cellulosomal enzymes were secreted and accordingly started to degrade Avicel (Fig. 2b, c). Therefore, it was suggested that a metabolic pathway seems to be different between the SBP and Avicel cultures (Fig. 5).

Shinohara et al. (2013) reported fixation of CO$_2$ in \textit{C. cellulovorans} by partly operated the TCA cycle in a reductive manner. In this study, \textit{C. cellulovorans} has been suggested to have a CO$_2$ fixation pathway, because of its ability to grow under a higher concentration of 100% CO$_2$ compared to other \textit{Clostridium} species. In the genome analysis of \textit{C. cellulovorans} (Tamaru et al. 2010), the genes of two important CO$_2$ fixation enzymes, namely pyruvate ferredoxin oxidoreductase (PFOR) and phosphoenolpyruvic acid (PEP) carboxylase (PEPC) were annotated. More interestingly, PFOR of glycolysis and PEPC of the TCA cycle are both in the node of main metabolic pathways in \textit{C. cellulovorans}. At this point, \textit{C. cellulovorans} produced 132 L/kg of H$_2$ and 190 L/kg of CO$_2$ under the cultivation of Avicel medium. Therefore, if these gases are completely converted to CH$_4$ through CO$_2$ reduction pathway in methanogens, more H$_2$ is theoretically required for CH$_4$ production.

Although much is not known of the mechanisms that create and maintain Methanosarcina diversity in any given environment, the distinct metabolism of the clade likely has a role (Youngblut et al. 2015). In addition, gene gain from bacterial taxa is common in at least some Methanosarcina spp. and may often be adaptive (Deppenmeier et al. 2002; Fournier and Gogarten)
Fig. 4 Cultivation of *C. cellulovorans*, CCeM and MFMP with SBP. **a** Total sugar concentration after 11-days cultivation in the culture with SBP, where negative control (open bar), CCeM (closed bar), MFMP (dotted bar) are included. **b** Cell growth in the culture of CCeM and MFMP with SBP, where CCeM (open circle), MFMP (closed circle). **c** Organic acid concentration in the CCeM (left) and MFMP (right) cultures with SBP, where lactic acid (Δ), acetic acid (*), butyric acid (black filled circle) are included. **d** Gas production after 11-days cultivation in the CCeM (left) and MFMP (right) cultures with SBP, where H₂ (closed bar), CH₄ (hatched bar), CO₂ (open bar) are included. Values indicate increments from the volume of negative control and are calculated as the volume per one kg of dry weight of SBP. **e** pH after 11-days cultivation with SBP, where negative control (open bar), *C. cellulovorans* (hatched bar), CCeM (closed bar), MFMP (dotted bar) are included. **f** Concentration of reducing sugar in the CCeM and MFMP cultures with SBP, CCeM (closed circle), MFMP (open circle). Values with error bars are mean ± SE of three independent samples. An asterisk indicates a significant difference (p < 0.05).
Host mobile element dynamics may also have a key role, given that *Methanosarcina* genomes contain a large number of putative mobile element genes and all contain multiple clustered regularly interspaced short palindromic repeats (CRISPRs) (Maeder et al. 2006; Nickel et al. 2013). Based on the 16S rRNA sequencing, *M. mazei* and the other methanogens were found in MFMP (Table 2, Fig. 3). In addition, various other miscellaneous microbes also existed. These results revealed that by using RLU as an index to construct the consortium, *C. cellulovorans* could survive with MFMP by setting the RLU ratio of *C. cellulovorans* and the MFMP that each of the initial RLUs was decided to 1 and 1000, respectively. In terms of CH$_4$ yield from SBP, 502.5 L/kg of CH$_4$ yields by using hydrothermal pretreatment and 360 L/kg by adding of external enzymes has been reported (Ziemiński et al. 2014; Miroslav et al. 2000). Although 34.0 L/kg of CH$_4$ yield in this study was lower than these reports, this study did not require any pretreatments and extra enzymes, suggesting that this study would have much advantages on a cost–benefit. In addition, since the yield depends on the saccharides concentration in SBP, the efficiency of sugar refinery in sugar factories would be able to control CH$_4$ yield. In fact, CH$_4$ production in the CCEM culture was lower than that in the MFMP culture, from another point of view, the volume reduction of SBP by *C. cellulovorans* is able to compensate the drying and transporting energy (Fig. 1). Furthermore, an adjusting the RLU ratio or pH in the CCEM culture are ways to improve CH$_4$ production. More interestingly, since the RLU value in the CCEM was extremely higher than the total value of the RLU value in the SBP monoculture and the MFMP culture (Fig. 2b), *C. cellulovorans* seems to interact with not only methanogens but also miscellaneous microbes. Therefore, there might have some possibilities that growing miscellaneous microbes in the CCEM increase their RLU and inhibit CH$_4$ production.

In future study, it could be possible to find various factors that are not gained from the coculture between *C. cellulovorans* and methanogens through omics analysis. Furthermore, by the machine learning using these data (Charlson et al. 2010), there are some possibilities that these omics data are able to elucidate not only inhibit factors for CH$_4$ production, but also interrelationship between each microbe in the CCEM.

**Authors’ contributions**

HT carried out all experiments and drafted the manuscript. FO discussed and suggested with the manuscript. YT (corresponding author) is responsible for this study, participated its design and help to draft the manuscript. All authors read and approved the final manuscript.

**Author details**

1 Department of Life Sciences, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya, Tsu, Mie 514-8507, Japan. 2 Department of Bioinformatics, Institute of Advanced Research Center, Mie University, 1577
References

Alam E, Mobin S, Chowdhury H (2015) Third generation biofuel from Algae. Procedia Eng 105:763–768

Bellido C, Infante C, Coca M, González-Benito G, Lucas S, García-Cubero MT (2015) Biochemical conversion processes of lignocellulosic from sugar beet pulp. Bioresour Technol 190:429–492

Brethauer S, Studer M (2015) Chemical conversion processes of lignocellulosic biomass to fuels and chemicals—a review. Chima 69:572–581

Charlson ES, Chen J, Custers-Allen R, Bittinger K, Li H, Sinha R, Hwang J, Bushman Brethauer S, Flierl RA (2010) Comparative genomics and evolution of the archaeal domain of life. Science 357(6351):eaaf3883

Chen J, Custers-Allen R, Bittinger K, Li H, Sinha R, Hwang J, Bushman Brethauer S, Flierl RA (2010) Comparative genomics and evolution of the archaeal domain of life. Science 357(6351):eaaf3883

Deppenmeier U, Müller V, Gottschalk G (1996) Pathways of energy conservation of anaerobic microbes. Arch Microbiol 165:149–163

Deppenmeier U, Johann A, Hartsch T, Merkl R, Schmitz RA, Martinez-Arias R, Henne A, Siezer A, Bäumer S, Jacoby C, Brüggemann H, Lienard T, Christmann A, Börneke M, Steckel S, Bhatthacharya A, Lykoldis A, Overbeek R, Klenke HP, Gnanalus RP, Fritz HJ, Gottschalk G (2002) The genome of Methanosarcina mazei: evidence for lateral gene transfer between bacteria and archaea. J Mol Microbiol Biotechnol 4:453–461

Doi RH, Kusugoe A (2004) Cellulose: plant-cell-wall-degrading enzyme complexes. Nat Rev Microbiol 2:541–551

Dredge R, Radloff, van Dyk JS, Pietichek B (2011) Lime pretreatment of sugar beet pulp and evaluation of synergy between ArMa, ManA and XynA from Clostridium cellulovorans on the pretreated substrate. 3 Biotech 1:511–519

Esaka K, Aburaya S, Morisaka H, Kuroda K, Ueda M (2015) Exoproteome analysis of Clostridium cellulovorans in natural soft-biomass degradation. AMB Express 5:2

Fournier GP, Gogarten JP (2007) Evolution of aceticlastic methanogenesis in methanosarcina via horizontal gene transfer from cellulolytic clostridia. J Bacteriol 190:1124–1127

Garcia JL, Patel BKC, Ollivier B (2000) Taxonomic, phylogenetic, and ecological diversity of methanogenic archaea. Anaerobe 6:205–226

Gray KA, Zhao L, Emptage M (2006) Bioethanol.Curr Opin Chem Biol 10:141–146

Guo J, Peng Y, Ni BJ, Han X, Fan L, Zhiguo Y (2015) Dissecting microbial community structure and methane-producing pathways of a full-scale anaerobic reactor digesting activated sludge from wastewater treatment by metagenomic sequencing. Microbiol Cell Fact 14:33

Hadden G, Klas H, Per Â (1986) The influence of wheat bran and sugar-beet pulp on the digestibility of dietary components in a cereal-based pig diet. J Nutr 116:242–251

Hattori S (2008) Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ 23:118–127

Koukiekolo R, Cho HY, Kusugoe A, Inai M, Yuka H, Doi RH (2005) Degradation of corn fiber by Clostridium cellulovorans cellulosomes and hemicellulases and contribution of cellulose binding protein Cbpa. Appl Environ Microbiol 71(7):3504–3511

Lu H, Ng SK, Jia Y, Lee PKH (2017) Physiological and molecular characterizations of the interactions in two cellulose-to-methane cocultures. Biotechnol Biofuels 10:37

Mairder DL, Anderson L, Breton TS, Brown DC, Gaila P, Han CS, Lapidus A, Metcalf WW, Sanders E, Tapia R, Sowers KR (2006) The Methanosarcina barkeri genome: comparative analysis with Methanosarcina acetivorans and Methanosarcina mazei reveals extensive rearrangement within methanosarcinal genomes. J Bacteriol 188:7922–7931

Matsuki K, Bae J, Esaka K, Morisaka H, Kuroda K, Ueda M (2013) Exoproteome profiles of Clostridium cellulovorans on various carbon sources. Appl Environ Microbiol 79:6576–6584

Miroslav H, Miloslav D, Mraflkova L (2000) Anaerobic biodegradation of sugar beet pulp. Biodegradation 11:203–211

Miya H, Maeya Y, Ishikawa T, Tanaka A (2016) Calorimetric studies of the growth of anaerobic microbes. J Biosci Bioengin 122(3):364–369

Naik SN, Goud VY, Rout PK, Dalabok AO (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sustain Energy Rev 14:578–597

Nakajima D, Shibata T, Tanaka R, Kuroda K, Ueda M, Miyake H (2017) Characterization of the cellulosomal scaffolding protein Cbpa from Clostridium cellulovorans 743B. J Biosci Bioengin 124:376–380

Nickel L, Weidenbach K, Jager D, Backofen R, Lange SJ, Heidrich N, Iizuka O, Hwang J, Bushman Brethauer S, Flierl RA (2010) Comparative genomics and evolution of the archaeal domain of life. Science 357(6351):eaaf3883

Schenk PM, Thomas-Hall SR, Stephens E, Manic UC, Mussgnug JH, Piren J, Krusek J, Christmann A, Börneke M, Steckel S, Bhatthacharya A, Lykoldis A, Overbeek R, Klenke HP, Gnanalus RP, Fritz HJ, Gottschalk G (2002) The genome of Methanosarcina mazei: evidence for lateral gene transfer between bacteria and archaea. J Mol Microbiol Biotechnol 4:453–461

Slet AE, Dhar RA, Robinson R (1984) Isolation and characterization of an anaerobic, cellulolytic bacterium, Clostridium cellulovorans sp. nov. Appl Environ Microbiol 48:888–93

Spang A, Gaces EF, Ettema TIG (2017) Genomic exploration of the diversity, ecology, and evolution of the archaeal domain of life. Science 357(6351):eaaf3883

Tomita Y, Urs S, Murashima K, Kusugoe A, Han CH, Doi RH, Liu B (2002) Formation of protoplasts from cultured tobacco cells and Arabidopsis thaliana by the action of cellulases and pectate lyase from Clostridium cellulovorans. Appl Environ Microbiol 68(5):2614–2618

Tamaru Y, Ueda M, Miyake H, Usui S, Murashima K, Kusugoe A, Han CH, Doi RH, Liu B (2002) Formation of protoplasts from cultured tobacco cells and Arabidopsis thaliana by the action of cellulases and pectate lyase from Clostridium cellulovorans. Appl Environ Microbiol 68(5):2614–2618

Tamaru Y, Ueda M, Miyake H, Usui S, Murashima K, Kusugoe A, Han CH, Doi RH, Liu B (2002) Formation of protoplasts from cultured tobacco cells and Arabidopsis thaliana by the action of cellulases and pectate lyase from Clostridium cellulovorans. Appl Environ Microbiol 68(5):2614–2618

Tomita Y, Miyake H, Kudou S, Ueda M, Doi RH (2010) Comparative genomics of the mesophilic cellulose-degrading Clostridium cellulovorans and its application to biofuel production via consolidated bioprocessing. Environ Technol 31:889–903

Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nat Rev Microbiol 6:579–591

Youngblut ND, Wirth JS, Henrikson JR, Smith M, Simon H, Metcalf WW, Whitaker RJ (2015) Genomic and phenotypic differentiation among Methanosarcina mazei populations from Columbia River sediment. ISME J 9(10):2191–2205

Zheng Y, Lee C, Yu C, Cheng YS, Zhang R, Jenkins BM, Vanderheuyden JS (2013) Dilute acid pretreatment and fermentation of sugar beet pulp to ethanol. Appl Energy 105:1–7

Ziemer K, Romanoviska L, Kowalska-Wentel M, Czyan M (2014) Effects of hydrothermal pretreatment of sugar beet pulp for methane production. Biosour Technol 166:187–193