Ph. D. Thesis

Studies on biofuel production from agricultural wastes by using *Clostridium cellulovorans*

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Contents

Abstract

For a resolution of reducing carbon dioxide emission and increasing food production to respond to the growth of global population, the production of biofuels from non-edible biomass is urgently required. Abundant agricultural wastes, such as orange wastes in orange juice factories and sugar beet pulp (SBP) from sugar refining factories, occur, but they are largely disposed of. These agricultural wastes are non-edible biomass and are able to be utilized as a raw material of second-generation biofuels. This study evaluates the isopropanol-butanol-ethanol (IBE) fermentation ability of *Clostridium beijerinckii* and cellulosic biomass degrading ability of *Clostridium cellulovoran*s under different concentrations of limonene, which has extremely toxic to fermenting microorganism. As a result, we found that *C. cellulovorans* was able to grow even in medium containing 0.05% limonene (v/v) and degraded 85% of total sugar from mandarin peel and strained lees without any pretreatments. More interestingly, *C. beijerinckii* produced 0.046 g butanol per 1 g of dried strained lees in the culture supernatant together with *C. cellulovorans*. Furthermore, this study indicated that a co-culture fermentation system combining *C. cellulovorans* with microbial flora of methane production (MFMP), using sugar beet pulp (SBP) as a carbon source, is effective for the direct conversion of cellulosic biomass to methane (CH_4). The MFMP was collected from a methane fermenter in commercial operation and was analyzed by a next-generation sequencing system. The microbiome was identified and classified based on several computer programs, revealing that the MFMP included methanogenic organisms such as, *Methanosarcina mazei*, *Methanosaetaceae*, *Methanosaeta* and *Methanospirillaceae*. Furthermore, Although the MFMP did not degrade SBP, the consortium of *C. cellulovorans* with MFMP (CCeM)

degraded 87.3% of SBP without any pretreatment and produced 34.0 L of CH_4 per 1 kg of dry weight of SBP. These results indicated that agricultural wastes can be degraded and converted to CH_4 simultaneously by *C. cellulovorans* and the MFMP.

1. Introduction

1.1 Background

Global warming, the rise in the average temperature of the Earth's climate system, is observed and its related impacts are concerned [1-3]. On the other hand, an atmospheric carbon dioxide concentration has increased remarkably in recent years, and the greenhouse effect of carbon dioxide is noted as a factor of global warming [4,5]. Therefore, the reduction of carbon dioxide emissions is an important topic. Although the use of fossil fuels emits much carbon dioxide, carbon dioxide discharged from biofuels is offset with carbon dioxide taken during plant growth. This results in the reduction of carbon dioxide emissions, and therefore many researches on biofuels, such as bioethanol [6,7], biodiesel [8], biochemicals [9] and economics of those [10], are ongoing [11,12]. Biofuels are shifting from first-generation biofuels made from corns and sugar canes, which compete with foods, to second-generation biofuels made from non-edible biomass [13-15]. Furthermore, development of third-generation biofuels made from algae has begun to be explored [16,17]. First- generation biofuels use starch from corn or sugar from sugar cane as a raw material, and the method of producing bioethanol is the same as liquor production, which has a long history. Therefore, first-generation biofuels, especially bioethanol, has been put to practical use and it has started to realize a low carbon society using carbon neutral materials. On the other hand, the global population is estimated to reach 9 billion in 2045 from 7 billion [18], and this population growth requires the increase of food production. Therefore, it is necessary to move on urgently from using food such as corn to non-edible biomass as a raw material, namely second-generation biofuels.

1.2 Cellulosic biomass

Cellulosic biomass, such as straw, corn stover and agricultural wastes, is non-edible biomass and is renewable. However, cellulosic biomass cannot be easily glycated like starch, and is largely disposed of. This is because cellulosic biomass is composed of cellulose, hemicellulose, pectin and lignin, which has rigid and complex structures [19]. Cellulose is comprised of a linear chain of D-glucose monomers bound together by β-1,4-glycosidic bonds and has a strong crystalline fiber structure [20], while hemicellulose consists of not only a monopolymer such as mannan and xylan, but also a heteropolymer such as arabinoxylan, glucuronoxylan, glucomannan, and xyloglucan. In addition, in cellulosic biomass lignin and phenol compounds are assembled with cellulose and hemicellulose [21]. Cellulose is a biopolymer consisting of many glucose units connected through β-1,4-glycosidic bonds, therefore the breakage of the β-1,4-glycosidic bonds by acids leads to the hydrolysis of cellulose polymers, resulting in the saccharides. Hydrochloric acid or sulfuric acid have been used in the hydrolysis of cellulose. However, they suffer from problems of product separation, reactor corrosion and the need for treatment of waste effluent. Therefore, environmental load becomes high [22,23]. These cellulose and hemicellulose are known to be degraded by enzymes such as cellulase, and the saccharification by enzymes occurs under low temperature and pressure conditions without hydrochloric acid or sulfuric acid. Therefore, the environmental load is much lower when using such enzymes compared with the hydrolysis by acid. Many microorganisms and fungi that secrete extracellular enzymes have been explored [24]. However, since the rigid and complex structures are constructed by cellulose, hemicellulose and lignin, it is not easy to degrade them enzymatically, especially with one enzyme alone. Researches on pretreatments, such as mechanical grinding, steam explosion and acid treatment, are being pursued for efficient degradation [25].

Furthermore, many enzymes, such as cellulases and hemicellulases, must cooperate together. [26].

1.3 Cellulosome

Some species of Clostridia are known to have the ability to degrade cellulosic biomass efficiently using a multiple-enzyme complex called the cellulosome together with non-cellulosomal enzymes [27-29]. The cellulosome is characterized by two major components, one is a scaffolding protein with multiple cohesin, which possesses dockerin-binding site, and the other consist of a variety of cellulosomal enzymes bound to dockerin. These components assemble into the cellulosome through the binding of cohesin and dockerin [30]. The cellulosome has cellulose-binding modules (CBMs), which combines with cellulose, so that the cellulosome can locate several enzymes close to cellulose (**Figure 1**). Furthermore, CbpA in the cellulosome is able to bind on a surface of the bacterium, facilitating the uptake of nearby degrading saccharides. There is not one combination of the enzymes on the cellulosome, but various combinations are possible. It is also known that various enzymes are closely located to each other and work synergistically. Additionally, Clostridia can produce non-cellulosomal enzymes, which are not connected with the cellulosome but often possess their CBMs. Clostridia are known to possess high cellulose degradation ability due to the cooperation of the cellulosome and such non-cellulosomal enzyme [31-37]. Research is also being conducted to construct artificial cellulosomes to promote the efficiency of biomass decomposition [38-40].

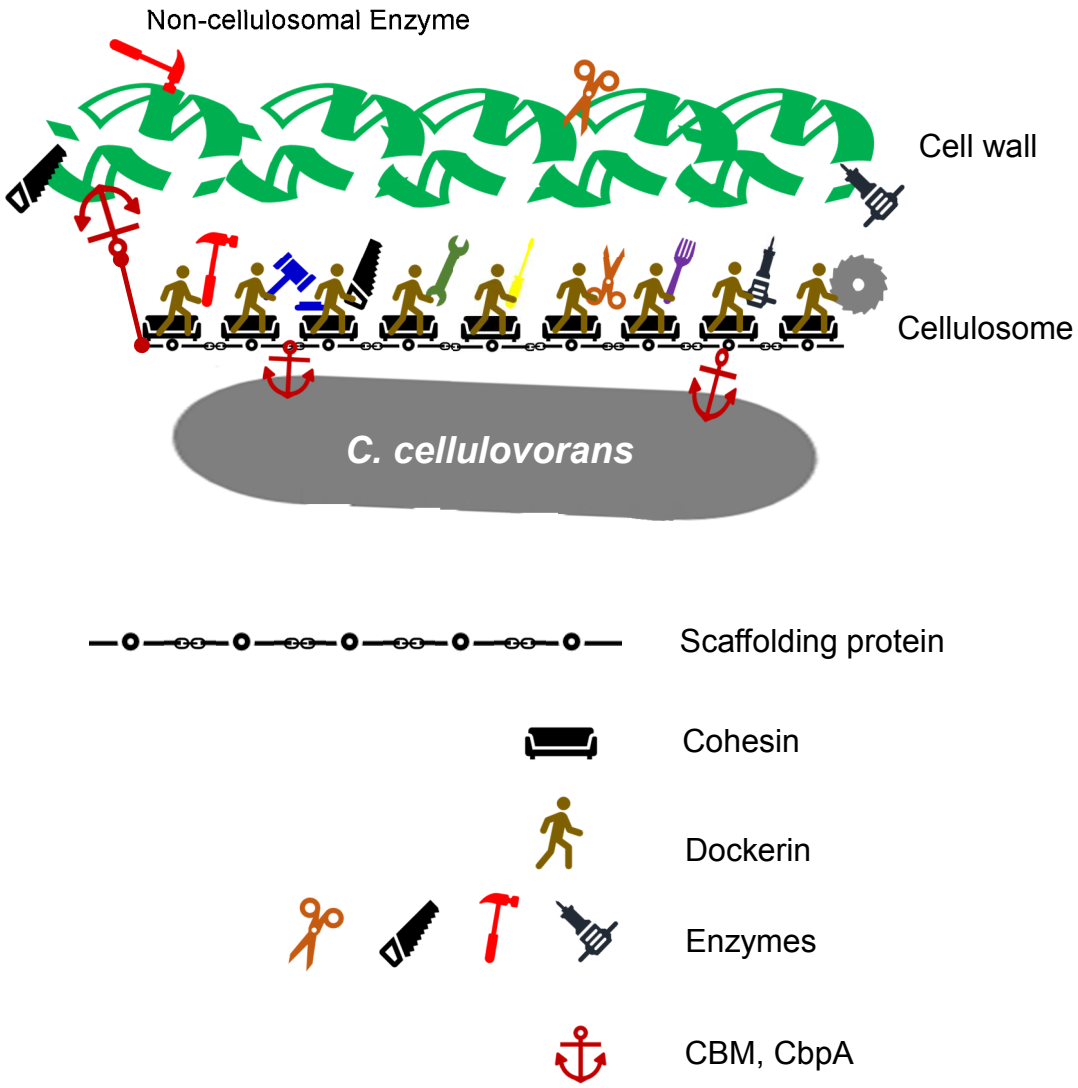


Figure 1. Schematic model of *C. cellulovorans* cellulosome.

1.4 *Clostridium cellulovorans*

Among those species, we have been studying *Clostridium cellulovorans*, which is an anaerobic, mesophilic, Gram-positive and spore-forming cellulolytic bacterium [41]. *C. cellulovorans* utilizes not only cellulose but also hemicellulose, such as xylan, fructan, galactan, and mannan, and pectin [42-45]. *C. cellulovorans* metabolizes formic acid, lactic acid, acetic acid, butyric acid, ethanol, CO_2 and H_2. The metabolized organic acid change by the culture condition [46] (**Figure 2**). As revealed by whole-genome sequencing of *C. cellulovorans*, 57 cellulosomal protein-encoding genes and 168 secreted-carbohydrase-encoding genes have been annotated [29,47]. A detailed characterization of *C. cellulovorans* was performed by Clostridia genome comparison [48]. Various cellulosomal enzymes from *C. cellulovorans* have been identified to date, which are a large gene cluster for CbpA-ExgS-EngH-EngK-HbpA-EngL-ManA-EngM-EngN [49,50], the endoglucanases EngB [51,52] and EngE [53], mannanase ManA [54], pectate lyase A [55], and the xylanases XynA [56] and XynB [57]. Thus, the cellulosomal enzymes from *C. cellulovorans* have high performance to degrade plant cell wall polysaccharides. This high degradation performance of *C. cellulovorans* has been reported previously, and several researches on the degradation mechanism for cellulosic biomass have been continued [58,59]. Furthermore, *C. cellulovorans* which was engineered metabolically by a transformation method produced biofuel directly from cellulose [60,61].

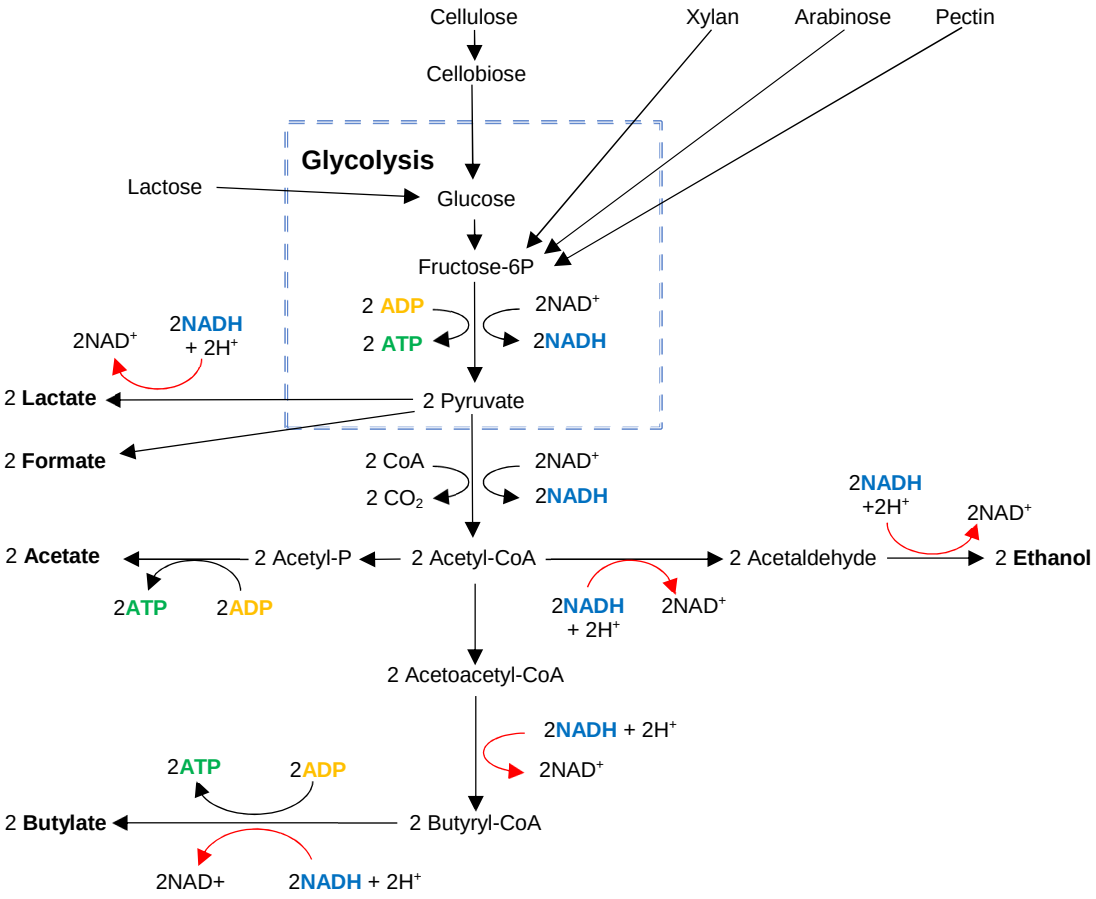


Figure 2. Central metabolic pathway in *Clostridium cellulovorans*, based on the data presented in references [40,60,61,62,123].

1.5 Agricultural wastes

Wheat, rice and corn are major crops and are cultivated all over the world. Their stovers are non-edible biomass and attractive candidates as a raw material of second-generation biofuels [63]. However, most of them are left uncollected in the field, and in order to use them as the raw material of biofuels, it is necessary to construct the collecting process. On the other hand, a lot of agricultural wastes are generated collectively at agricultural processing factories, such as orange wastes in an orange juice factory and sugar beet pulp in a sugar refining factory. These agricultural wastes are available without the new collection process from fields, and it has the potential to be an initiator to disseminate second-generation biofuels.

1.5.1 Orange wastes

Orange juice is one of the major fruit juices and and 1.6 million metric tons are produced per year around the world [64]. Almost the same amount of orange wastes come out as by-product from the orange juice factories. These orange wastes are available non-edible biomass. Part of these are used as animal feed, but a large proportion of these have to be disposed of due to high drying and transportation costs [65]. The orange wastes, such as peel and strained lees, still contain much sugar, which conventional yeast, such as *Saccharomyces cerevisiae,* can utilize to ethanol fermentation [66]. Also, useful chemical components can be extracted from citrus wastes [67]. *S. cerevisiae* can be engineered for simultaneous maltose utilization and in-situ carbon dioxide (CO_2) fixation to achieve efficient xylose fermentation [68]. However, D-limonene, hereafter called limonene, which is included in citrus fruits, is reported to have extreme toxicity to fermenting microorganism [69,70,71]. Therefore, the fermentation with *S. cerevisiae* requires prior separation of limonene from the medium, or to protect from it by an

encapsulation or an immobilization [72,73]. On the other hand, few studies have been reported on the fermentation from citrus fruits by *Clostridium beijerinckii*. *C. beijerinckii* are well-known for isopropanol-butanol-ethanol (IBE) fermentation ability and are employed repeatedly for research from the early 20th century [74] (**Figure 3a, b**). *C. beijerinckii,* which is also a mesophilic and anaerobic bacterium, is known to assimilate monosaccharides such as glucose, xylose, mannose and arabinose, and to convert them into organic acid such as acetic acid, lactic acid and butyric acid, and alcohols such as, butanol, isopropanol and ethanol [75,76]. Furthermore, *C. beijerinckii* achieved the solvent productivity of 5.52 g/L/h, with the yield of 54% from glucose with wood pulp as a cell holding material [77].

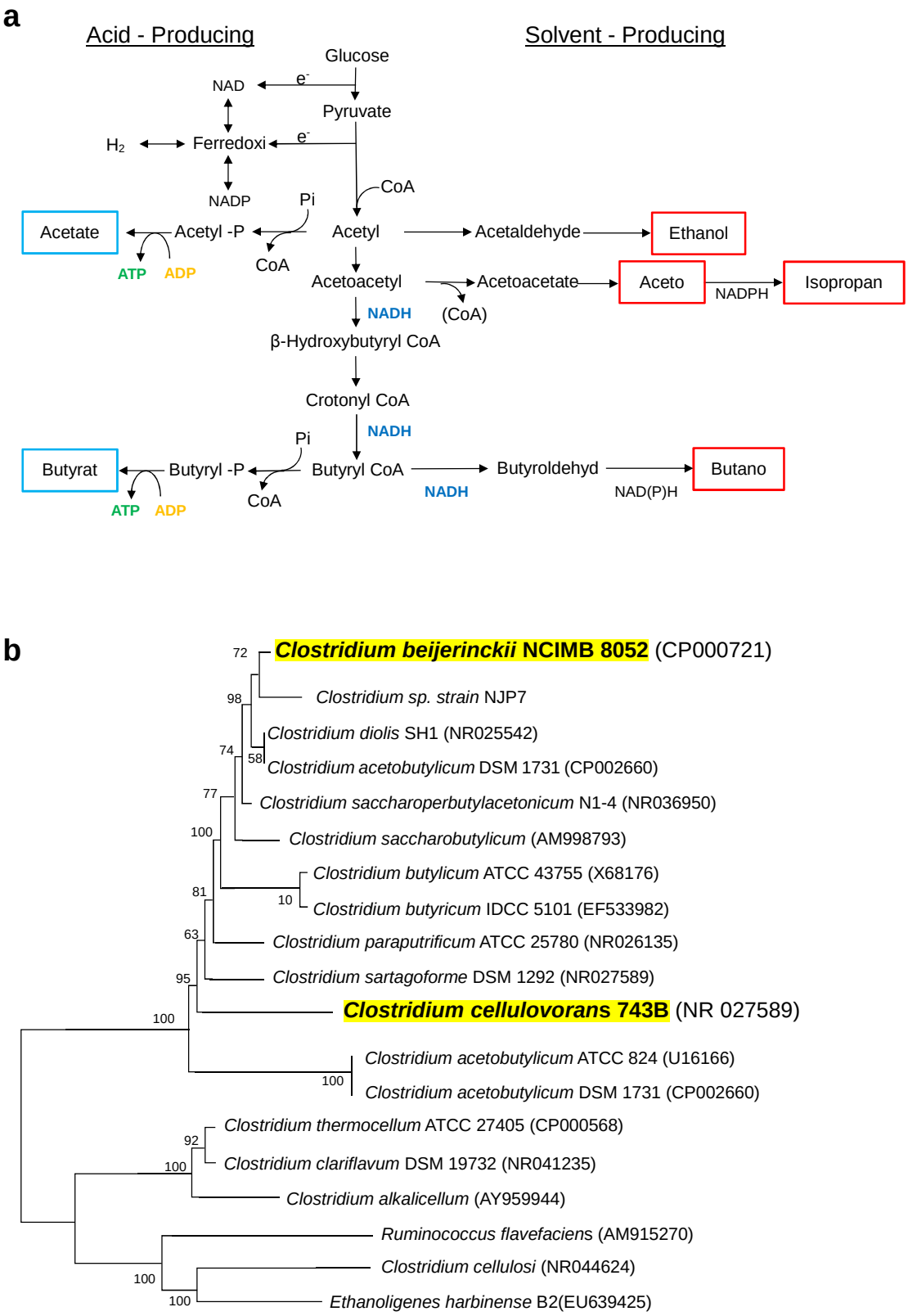


Figure 3. (**a**) Metabolic pathway for acetone−isopropanol−butanol−ethanol (IBE) production within *Clostridium* spp. [78]. (**b**) Phylogenetic tree of *Clostridium* spp. [79].

1.5.2 Sugar beet pulp

Sugar is essential for human beings and about 20% of the world's sugars is supplied by the root of a sugar beet (*Beta vulgaris* L.), which are cultivated all over the world, but mostly in Europe, North America and Russia [80]. Sugar beet pulp (SBP) is a by-product of the sugar production from 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). The pulp is then mashed by heating with water 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 SBP is the residue and non-edible biomass, it is the subject of research as a raw material of second-generation biofuels [81,82]. 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 pretreatment processes are not necessary to remove lignin (**Table 1**).

Table 1. Chemical components of SBP

Component	Weigh (g) per dry matter (100g)			
	Hadden et al. (1986) [83]		Zheng et al. (2013) [82]	
Ash	3.42	g/100g	2.51	g/100g
Proteins	11.42	g/100g	11.42	g/100g
Lipids	1.63	g/100g	-	
Sugars[*1]	5.2	g/100g	-	
Starch	0.99	g/100g	-	
Lignin	2.38	g/100g	1.16	g/100g
Glucan	17.34	g/100g [*2]	22.7	g/100g
Xylan	1.36	g/100g [*2]	5.14	g/100g
Galactan	4.88	g/100g [*2]	5.92	g/100g
Arabinan	16.83	g/100g [*2]	23.73	g/100g
Mannan	1.58	g/100g [*2]	1.85	g/100g
Pectin	21.15	g/100g [*2]	22.84	g/100g
Others		-	2.73	g/100g

[*1] Total value of rest of fructose, glucose, sucrose and fructan.

[*2] Conversion of values to polysaccharides in the paper.

1.5.3 Sorghum

The search for biomass suitable for biofuels besides agricultural wastes is in progress, with *Sorghum bicolor*, hereafter sorghum, being one of candidates [84-88]. Sorghum belongs to Poaceae and grows fast, reaching four meters tall, utilizing C_4 carbon fixation (NADP-ME type). Therefore, large amounts of biomass can be expected from this plant [89]. It has relatively low input requirements with the ability to grow on marginal lands. The seed of sorghum is gluten free and can be used instead of flour, and the squeezed juice from the stalk is rich in sugar. The remaining stalks and leaves can be utilized as a raw material for biofuels.

1.6 Methanogenesis

First-generation biofuels are mainly bioethanol production [13]. If intended to replace gasoline, liquid biofuel production, such as bioethanol, is be required. However, methane is an option, if gaseous replacement biofuels are required in agricultural processing factories, such as sugar refining factories. Methane fermentation is a conventional way to generate biofuels, and has been studied for a long time [90-92]. Many reports have been made across a wide range of academic fields [93-95]. The activity of decomposing biomass and producing methane as a final product is widely practiced in the natural world, and has been reported even from the permafrost. This suggests methane production to be one of the most robust and sustainable processes on Earth [96]. Methane fermentation proceeds with three metabolic groups of bacteria. Fermentative bacteria hydrolyze materials such as polysaccharides, lipids and protein, and excreted acetate and other saturated fatty acids, CO_2 and H_2 as major end products. A second group produces acetate and H_2 from end-products of the first group. The last group, which are methanogenic bacteria, catabolize mainly acetate, CO_2 and H_2 produced jointly by the other two groups, to the terminal products, such as CH_4, CO_2 and H_2O [97]. There are two major methane producing pathways, one being the CO_2 reduction pathway, in which CH_4 is produced from H_2 and CO_2, and another where CH_4 is produced from CH_3COOH. Apart from that, CH_4 is produced by syntrophic formate oxidation coupled with CO_2 reduction and formate methanogenesis under anaerobic conditions. [98].

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$

$$4HCOOH \rightarrow CH_4 + 2H_2O + 3CO_2$$

Which methane production pathway works is decided by the type of methanogenic bacteria, the interaction between metabolic groups and the culture condition. Methane production is carried out by the complex microbial flora including methanogens, and it has been formerly difficult to grasp the microbial flora comprehensively. However, it has now become possible to analyze the whole aspect of the microbiome characteristics using the next-generation sequencing system [99]. Clostridia accelerates methanogenesis [100]. This has been reported as a result of coculturing *C. cellulovorans* with one of the famous methanogens such *Methanosarcina* spp. [62]. Since *C. cellulovorans* and methanogens were both able to grow anaerobically under mesophilic conditions, it was possible to cultivate them in a single tank and therefore simultaneously degrade cellulosic biomass and produce methane (CH_4). However, there are few reports on a consortium of *C. cellulovorans* and microbial flora of methane production (MFMP).

1.7 The purpose of the present studies

In order to effectively use orange wastes, this study demonstrates the tolerance of *C. beijerinckii* and *C. cellulovorans* against different concentrations of limonene. The IBE fermentation ability of *C. beijerinckii* and cellulose degradation ability of *C. cellulovorans* was evaluated in the culture medium including mandarin peel and strained lees as sole carbon sources. This study used mandarin oranges because mandarin oranges are popular in Japan and have limonene as same as other citrus fruits. Furthermore, processes for producing CH_4 and hydrogen (H_2) via the co-culture of *C. cellulovorans* with microbial flora of methane production (MFMP), (called the Consortium of *C. cellulovorans* with the MFMP (CCeM)), with carbon sources such as SBP, mandarin orange wastes, sorghum bicolor and Avicel, are being investigated. First, we analyzed 16s 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 as well as the CCeM co-culture were carried out to evaluate concentrations of sugars, organic acid, and biogas (H_2 and CH_4) yield after cultivation.

2. Material & Method

2.1 Culture condition of cultivation

Saccharomyces cerevisiae BY4741 was used and precultured anaerobically in YPD media with 2.0 % (w/v) glucose (Wako, Osaka, Japan) at 30 °C for 72 h without shaking. YPD media was used for one liter of medium: 10 g of Yeast extract (Bacto, MD, USA), 20 g of Pepton (Bacto), 20 g of Glucose (Wako). pH was adjusted 6.

Clostridium cellulovorans 743B (ATCC 35296) was used and precultured anaerobically in the media with 0.5% (w/v) cellobiose (Sigma, MO, USA) at 37 °C without shaking. *Clostridium beijerinckii* NCIMB8052 (ATCC 51743) was used and precultured anaerobically in the media with 2.0% (w/v) glucose (Wako) at 37 °C without shaking. *Clostridium cellulovorans* medium was partially modified and used [41]. Per one liter of medium was prepared with 4 g of Yeast extract, 1 mg of Resazurin salt, 1 g of L-Cysteine HCl, 5 g of $NaHCO_3$, 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.3675 g of NH_4Cl, 0.9 g of NaCl, 0.1575 g of $MgCl_2.6H_2O$, 0.12 g of $CaCl_2.2H_2O$, 0.85 mg of $MnCl_2.4H_2O$, 0.942 mg of $CoCl_2.6H_2O$, 5.2 mg of Na_2EDTA, 1.5 mg of $FeCl_2.4H_2O$, 0.07 mg of $ZnCl_2$, 0.1 mg of H_3BO_3, 0.017 mg of $CuCl_2.2H_2O$, 0.024 mg of $NiCl_2.6H_2O$, 0.036 mg of $Na_2MoO_4.2H_2O$, 6.6 mg of $FeSO_4.7H_2O$, 0.1 g of p-aminobenzoic acid, and was adjusted to pH 7 for *C. cellulovorans* and pH 5 for *C. beijerinckii*, respectively.

The MFMP was obtained from methane fermentation digested liquid collected 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 without shaking.

2.2 Preparation of substrates

Mandarin oranges purchased at a grocery store were used. Flavedo and albedo, hereafter called removed peel, were removed before squeezing (**Figure 4a**). Whole mandarin oranges except removed peel were squeezed by a squeezing device, hereafter called strained lees (**Figure 4b**). Mandarin oranges were squeezed by SJC-75-W (Irisohyama, Miyagi, Japan). The substrate concentrations of removed peel and strained lees were 1.0% (w/v) of dry weight. 10 vials of a medium containing removed peel and 10 vials of a medium containing strained lees were prepared. SBP was obtained from a sugar factory in Hokkaido, Japan. Sorghum cultivated in Aichi, Japan was used (**Figure 4c**). SBP and sorghum were dried up, milled and sieved through 80 meshes. The substrate concentrations of SBP, sorghum and Avicel (Sigma, MO, USA) were 0.5% (w/v) of dry weight. Limonene (Wako) was used for different concentrations media.

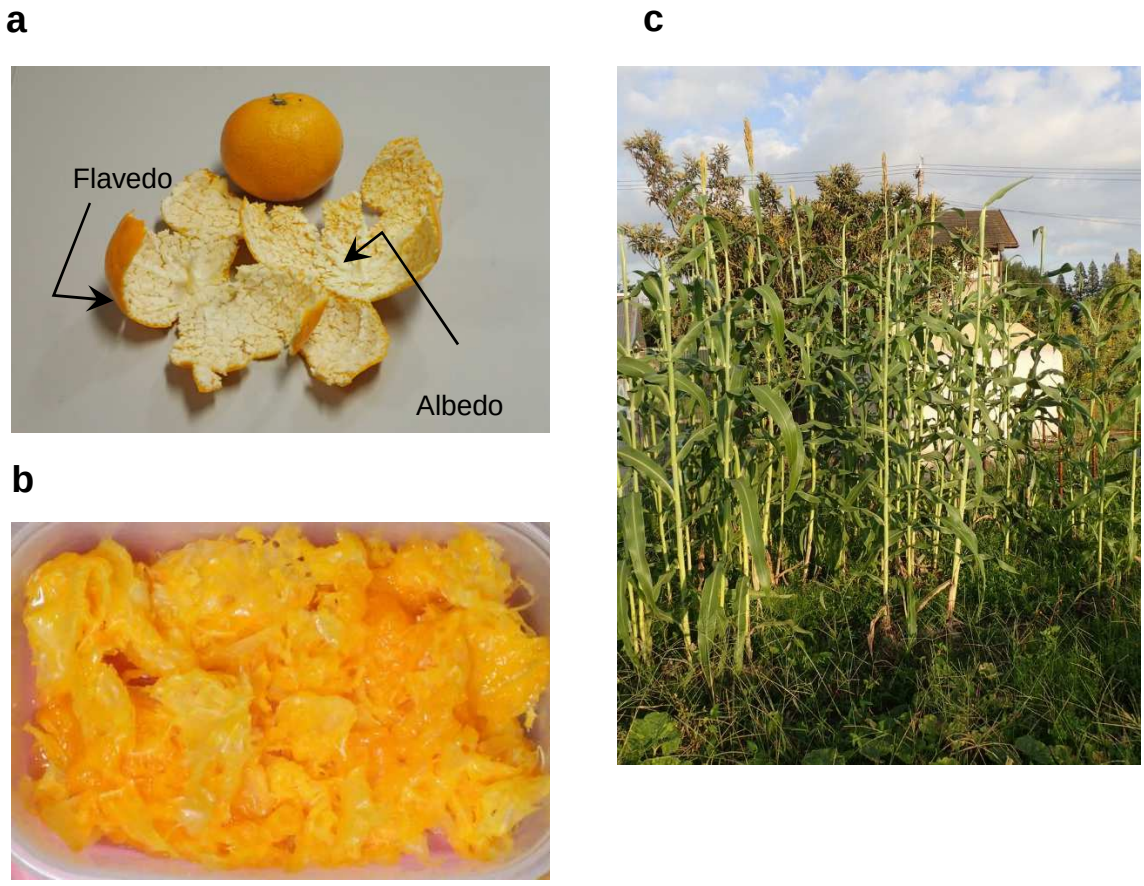


Figure 4. (**a**) Flavedo and albedo are removed before squeezing and (**b**) after squeezing (strained lees). Sugar Drip Sorghum (**c**).

2.3 Measurement of total sugar and reducing sugar concentrations

Total sugar concentration was measured by Phenol-sulfuric acid method. Reducing sugar was measured by DNS method (Dinitrosalicylic Acid), as D-glucose equivalents [101].

2.4 Alcohol concentration

Alcohol concentration was measured by a gas chromatograph GC-2010plus (Shimadzu, Kyoto, Japan) with FID detector and a capillary column Rt-Q-BOND (30 m, inner diameter. 0.32 mm; RESTEK, PA, USA). The oven temperature was 250°C and the column temperature was 150°C. Nitrogen was the carrier gas and set at a flow rate of 1.21 mL/min.

2.5 Organic acid concentration

The concentration of organic acid 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. The injection volume of each sample was 20 μL.

2.6 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 concentrations of methane, hydrogen, and carbon dioxide were 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. The injection volume of each sample was 5 ml.

2.7 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 x 300bp. Qiime as an analyzing software and Greengene as a database were used, and OTU was decided except chimeric genes.

2.8 Data deposition

The sequences reported in this paper have been deposited in the DDBJ database (accession104 no. DRR160954).

2.9 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 [102]. Cell growth was estimated by measuring ATP concentration of 0.1 ml of cell culture according to the manufacturer’s instruction and was expressed by Relative Light Unit (RLU) value.

2.10 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.

3. Results

3.1.1 Ethanol fermentation and glucose concentration with *Saccharomyces cerevisiae* under different concentrations of limonene

Anaerobic batch cultivations of *S. cerevisiae* were carried out in a 30-ml YPD medium containing 2% glucose with 0, 0.01, 0.02, 0.05 and 0.1% (v/v) limonene at 30 °C without shaking. Concentrations of ethanol and glucose were measured at 24- and 48-h cultivation, respectively. Whereas ethanol fermentation was inhibited under more than 0.02% of limonene (**Figure 5a**), glucose consumption was increased under up to 0.02% of limonene (**Figure 5b**). Furthermore, ethanol concentration at 48-h cultivation was significantly lower when cultured with more than 0.02% of limonene (**Figure 5d**). Although cell growth at 0-h, or just after inoculation, did not show significant differences, it was inhibited significantly with more than 0.01% limonene after 48-h cultivation (**Figure 5c**).

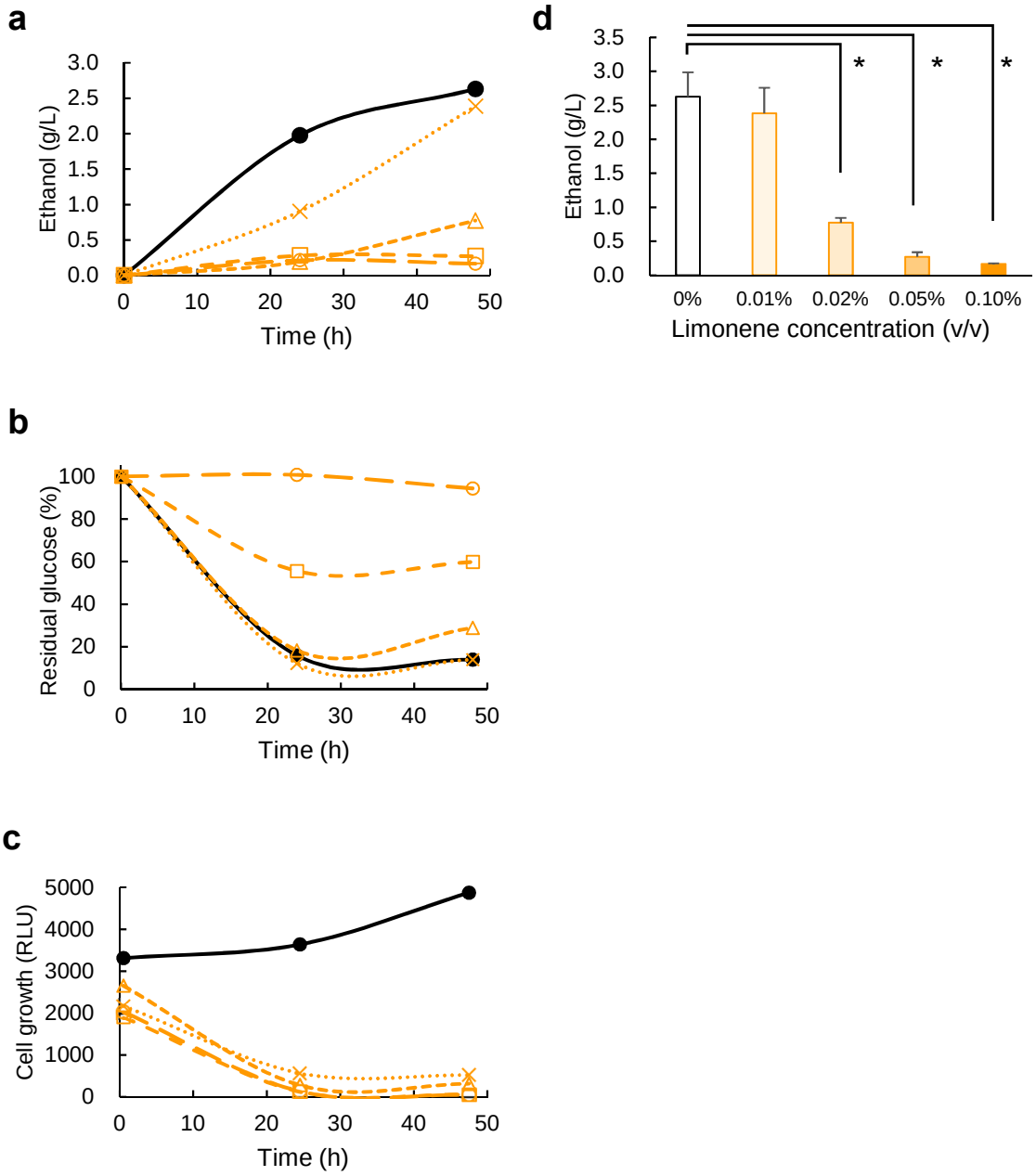


Figure 5. Concentration of ethanol (**a**), residual glucose ratio (**b**) and cell growth (**c**) in the culture medium with *S. cerevisiae*, where different concentrations of limonene (v/v), 0% (●_black filled circle), 0.01% (×), 0.02% (△), 0.05% (□), 0.1% (○_open circle), were present in the culture medium. **d** Ethanol production at 48- h cultivation. Values are means ± SE of three independent samples. An asterisk indicates a significant difference ($p<0.05$).

3.1.2. IBE fermentation and glucose concentration with *C. beijerinckii* under different concentrations of limonene

Anaerobic batch cultivations of *C. beijerinckii* were carried out in a 30-ml medium containing 2% glucose with 0, 0.01, 0.02, 0.05 and 0.1% (v/v) limonene at 37 °C without shaking. Alcohol and glucose concentrations were measured at 48- and 72-h cultivations, respectively. Total values of ethanol, isopropanol and butanol concentration were taken as alcohol concentration. Alcohol production was decreased on 0.05% limonene at 48-h cultivation, but was finally increased at 72-h cultivation (**Figures 6a**). On the other hand, glucose consumption showed a similar pattern and reached to about 50% decrease of initial glucose concentration except in 0.1% limonene (**Figure 6b**). In comparison under different concentrations of limonene at 72-h cultivation, alcohol fermentation by *C. beijerinckii* was completely inhibited under 0.1% limonene (**Figure 6d**). These results indicated *C. beijerinckii* could ferment glucose to alcohol under less than 0.05% limonene and limonene tolerance of *C. beijerinckii* was five times higher than that of *S. cerevisiae*. Interestingly, cell growths in the culture media with limonene at 0- and 20-h cultivation hardly increased, however cell growth with less than 0.2% limonene turned to increase at 24-h cultivation (**Figure 6c**).

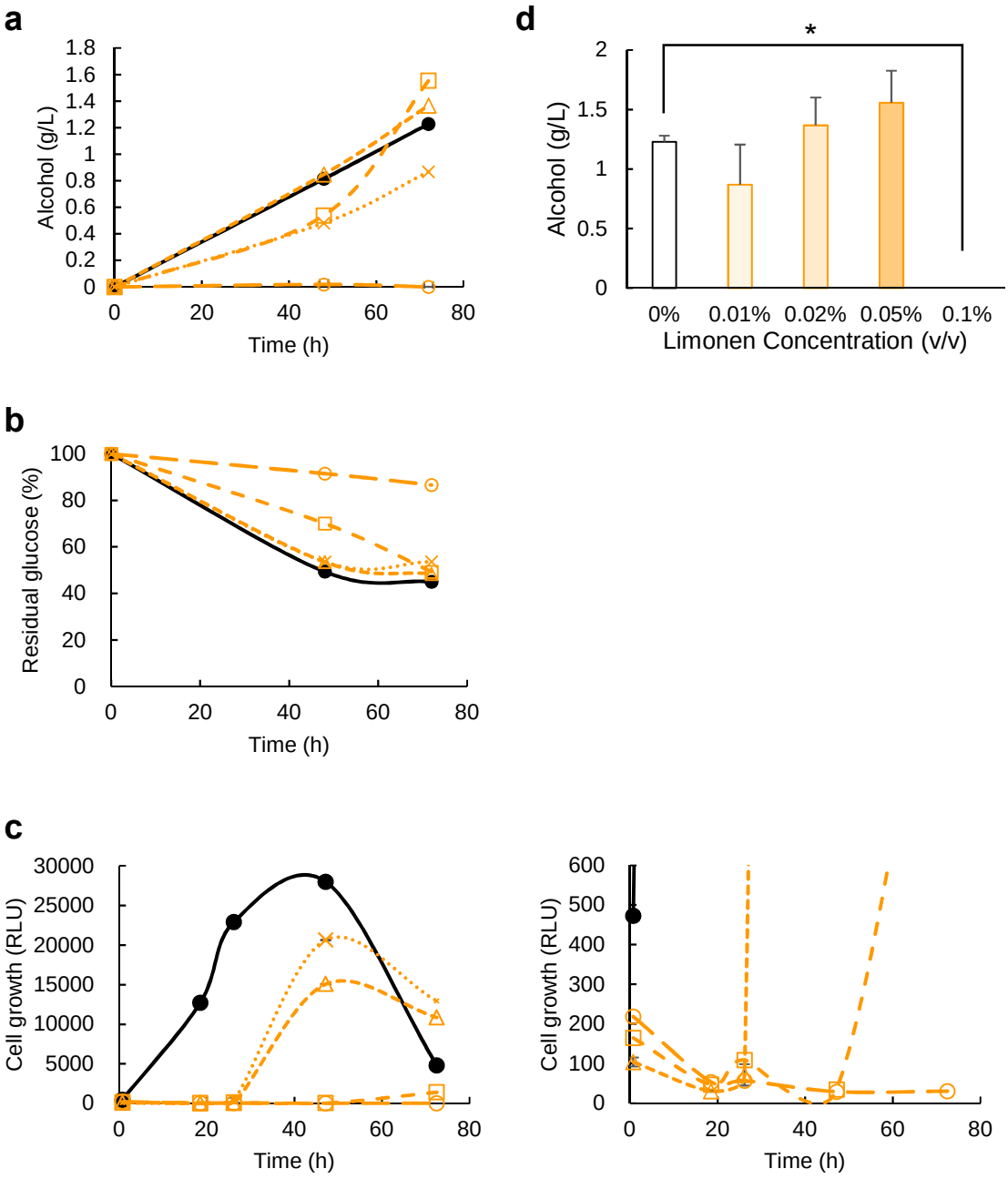


Figure 6. Concentration of alcohol (**a**), residual glucose ratio (**b**) and cell growth (**c**) in the culture medium with *C. beijerinckii*, where different concentrations of limonene (v/v), 0% (●_closed circle), 0.01% (×), 0.02% (△), 0.05% (□), and 0.1% (○_open circle), were present in the culture medium. **d** Alcohol production at 72-h cultivation. Values are means ± SE of three independent samples. An asterisk indicates a significant difference ($p<0.05$).

3.1.3. Cellulose degradation with *C. cellulovorans* under different concentrations of limonene

Anaerobic batch cultivations of *C. cellulovorans* were carried out in a 30-ml medium containing 0.5% Avicel with 0, 0.01, 0.02, 0.05 and 0.1% (v/v) of limonene at 37 °C without shaking. Total sugar concentration was measured at 8-, 26-, 39- and 61-days cultivation, respectively. Whereas Avicel was completely degraded by *C. cellulovorans* without limonene (0%) at 39-days cultivation, approximately 60% was degraded with 0.01-0.05% limonene (**Figures 7a**). After 61 days cultivation, Avicel was almost completely degraded in the presence of 0.01-0.05% limonene. On the other hand, Avicel was degraded even in 0.1% limonene according to the measurement of total sugar concentration. As a result, there was not a significant difference in comparison with the control (without limonene) (**Figure 7d**). Interestingly, *C. cellulovorans* survived for 2 months, even though there was less carbon source for *C. cellulovorans* at earlier stages of cultivation. It was suggested that *C. cellulovorans* was able to survive by secreting cellulosome and non-cellulosomal enzymes, taking the saccharide from degrading Avicel. Furthermore, surprisingly, cell growth in the culture with limonene at 0-h, or just after inoculation, was extremely low compared to 0% limonene. The RLU levels were almost comparable to negative controls (**Figure 7b, c**). From the fact that intracellular ATP decreased drastically, it was indicated that *C. cellulovorans* sensed trace amount of limonene (0.01%) and intracellular ATP was discharged rapidly. However, cell growth in the culture with limonene turned to increase, and cell growth started to increase early in the medium with low limonene concentration.

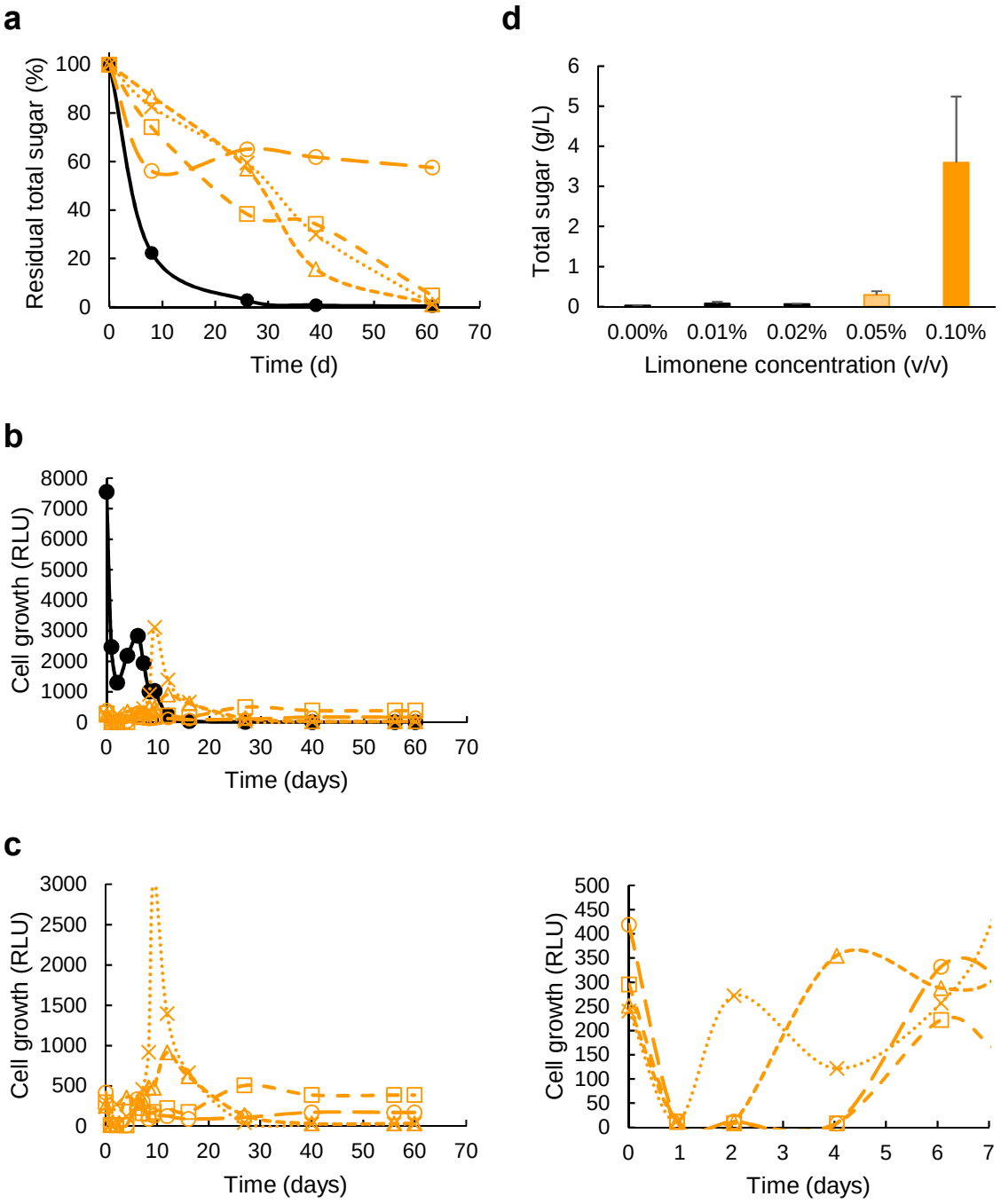
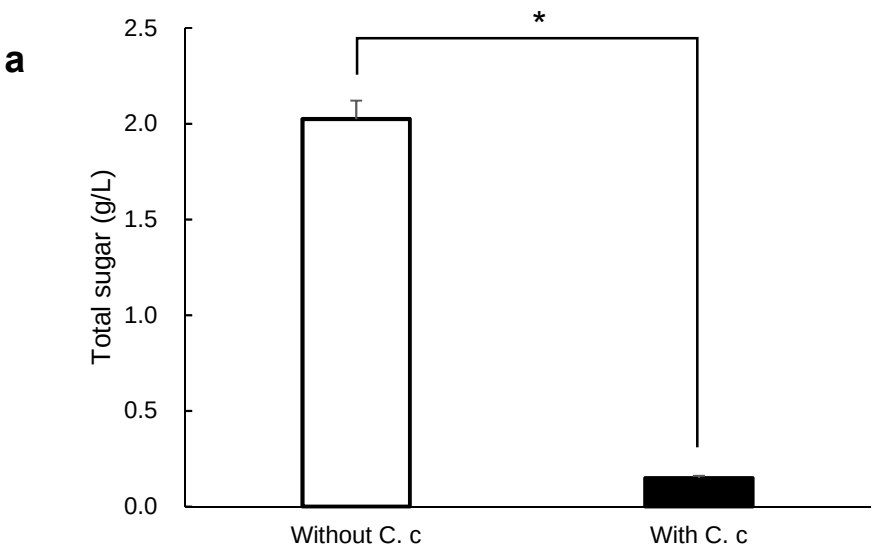
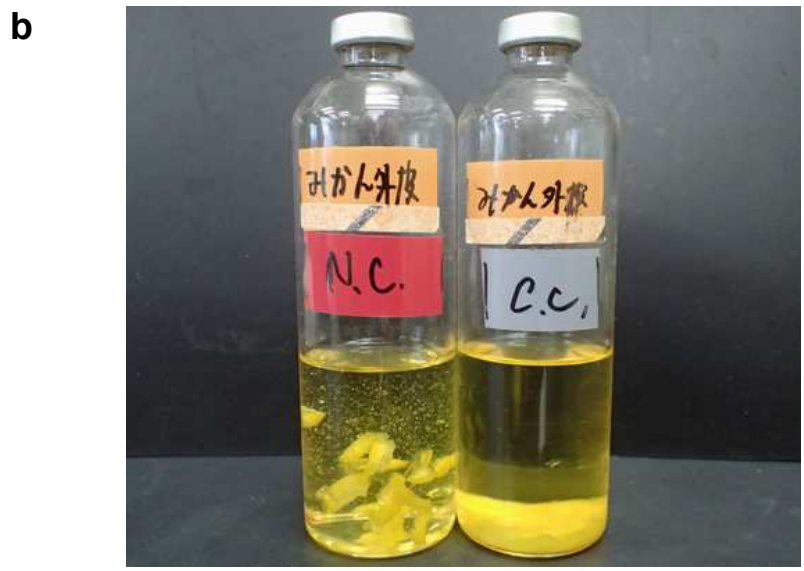


Figure 7. Residual total sugar ratio (**a**), cell growth (**b, c**) in the culture with *C. cellulovorans*, where different concentrations of limonene (v/v), 0% (●_closed circle), 0.01% (×), 0.02% (△), 0.05% (□), 0.1% (○_open circle), were present in the culture medium. **d** Total sugar concentration at 61-days cultivation. Values are means ± SE of three independent samples.

3.1.4. Degradation of mandarin orange peel and strained lees with *C. cellulovorans*

The removed peel was put in a 15-ml vial placed on an electronic scale and the weight was measured except tare. Dry weight was calculated from the water content, which was 71.6%. The removed peel was added into *C. cellulovorans* medium as 1% (w/v) of a dried substrate. The final volume of the medium as approximately 2 ml for each vial. 10 vials were made. The other 10 vials of the medium containing strained lees were made similarly, in accordance with 83.9% water content. Five vials each were inoculated with 0.2 ml of preculture medium containing 0.5% cellobiose with *C. cellulovorans* for both removed peel and strained lees media. All vials were cultivated at 37 °C without shaking. The culture supernatant was removed after centrifugation and total sugar of culture residues was measured after 16-days cultivation. Total sugar in the removed peel media with or without *C. cellulovorans* were 0.148 g/L and 2.025 g/L, respectively (**Figure 8a, b**), while total sugar in the strained lees media with or without *C. cellulovorans* were 0.241 g/L and 1.654 g/L, respectively (**Figure 9a, b**). These results indicated *C. cellulovorans* degraded 93% of removed peel and 85% of strained lees, respectively, without any pretreatment of these substrates.





Without *C. cellulovorans* With *C. cellulovorans*

Figure 8. (**a**) Total sugar concentration in the culture medium containing removed peel. Values are means ± SE of three independent samples. An asterisk indicates a significant difference ($p<0.05$). (**b**) The media were with and without inoculation of *C. cellulovorans* after cultivation.

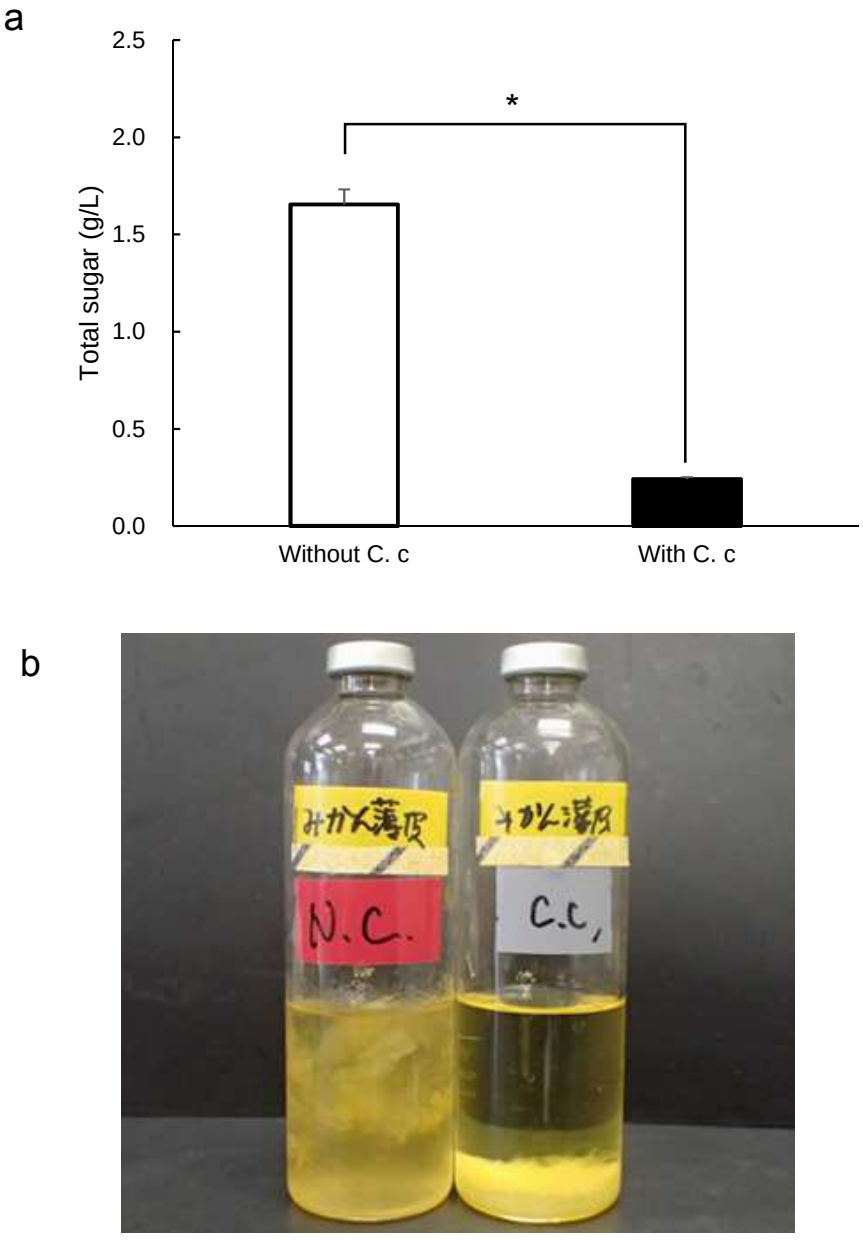


Figure 9. (**a**) Total sugar concentration in the culture medium containing strained lees. Values are means ± SE of three independent samples. An asterisk indicates a significant difference ($p<0.05$). (**b**) The media were with and without inoculation of *C. cellulovorans* after cultivation.

3.1.5. IBE fermentation by *C. beijerinckii* from the culture supernatant with *C. cellulovorans*

0.1 ml of preculture medium of *C. beijerinckii* was inoculated in 1 ml of supernatant taken from 16-days culture of *C. cellulovorans*, and they were then cultivated at 37 °C without shaking. Butanol concentration was measured at 18-days cultivation. The measured values of butanol concentration were multiplied by the volume of each vial medium and the weight of butanol per vial was calculated. The calculated butanol weight was divided by the dry weight of each vial substrate as a final yield. Butanol yield from strained lees cultivated with *C. cellulovorans* was twice as higher than that without *C. cellulovorans* (**Figure 10**). Namely, the maximum yield of butanol was 0.046 g per 1 g of the strained lees in the supernatant with *C. cellulovorans*. In contrast, butanol yield was 0.005 g per 1 g of removed peel in the supernatant without *C. cellulovorans*. Moreover, the cultivation conditions were compared with before or after addition of *C. beijerinckii* to the cultivated media with or without *C. cellulovorans*. As a result, reducing sugar in the culture supernatants after addition of *C. beijerinckii* were always lower than before addition (**Figure 11**). In particular, in the case of removed peel as a substrate without *C. cellulovorans* and before addition of *C. beijerinckii*, the reducing sugar concentration was highest among all the conditions. These results suggested that sugar components for IBE fermentation of *C. beijerinckii* might be different between removed peel and strained lees. All concentrations of organic acid in the culture supernatants after addition of *C. beijerinckii* were higher than before addition of that except butyric acid concentration in the strained lees culture (**Figure 12**). Butyric acid concentration in the strained lees culture was not significantly different between with and without *C. cellulovorans*. It was suggested that there was a lot of saccharides, which *C. beijerinckii* was able to consume, in the strained lees culture. *Clostridium* species produce acetate and butyrate after the

metabolism shifts to the solvent production phase. Thus, it was also suggested that *C. beijerinckii* in the strained lees culture produced higher concentration of butanol than the removed peel culture by utilizing those rich saccharides and shifting the solvent production phase (**Figure 10, 11**).

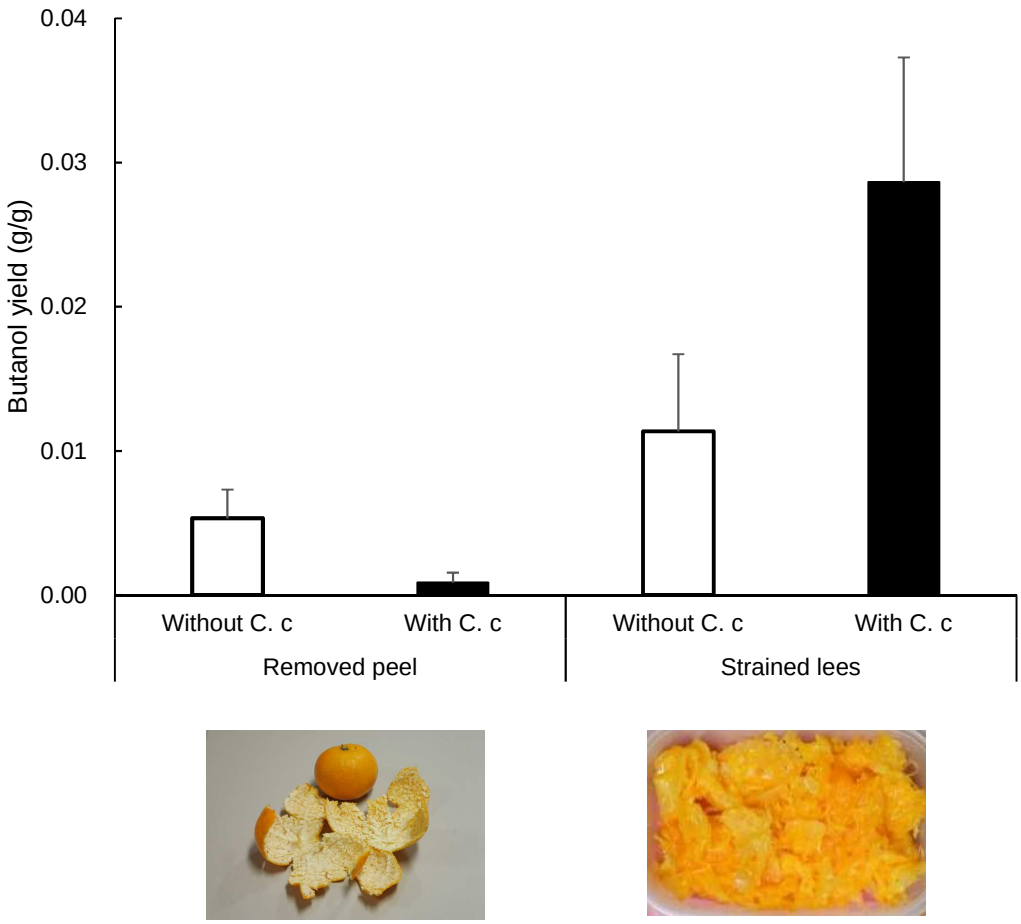


Figure 10. Butanol concentration in the culture supernatants with *C. beijerinckii* from removed peel and strained lees with or without *C. cellulovorans*. Values are means ± SE of four independent samples.

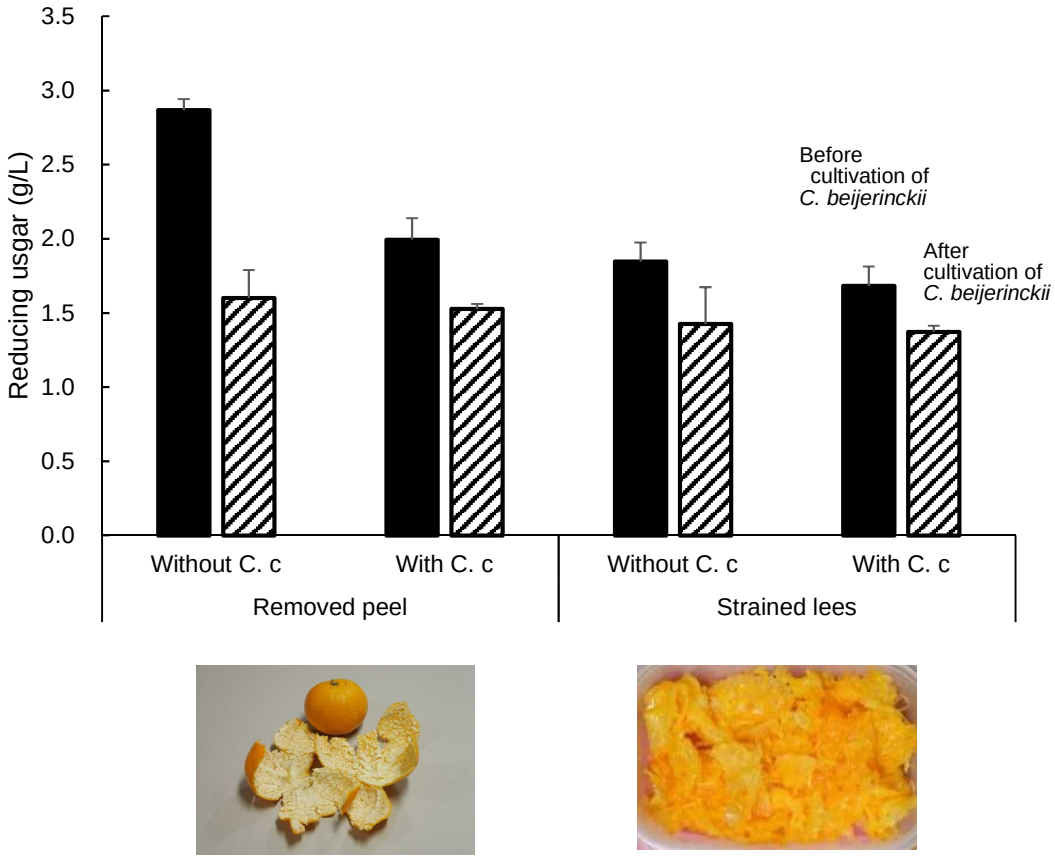


Figure 11. Reducing sugar concentration in the culture supernatant from removed peel and strained lees with or without *C. cellulovorans*. Closed bars and hatched bars indicate before addition of *C. beijerinckii* and after addition *C. beijerinckii*, respectively. Values are means ± SE of five independent samples.

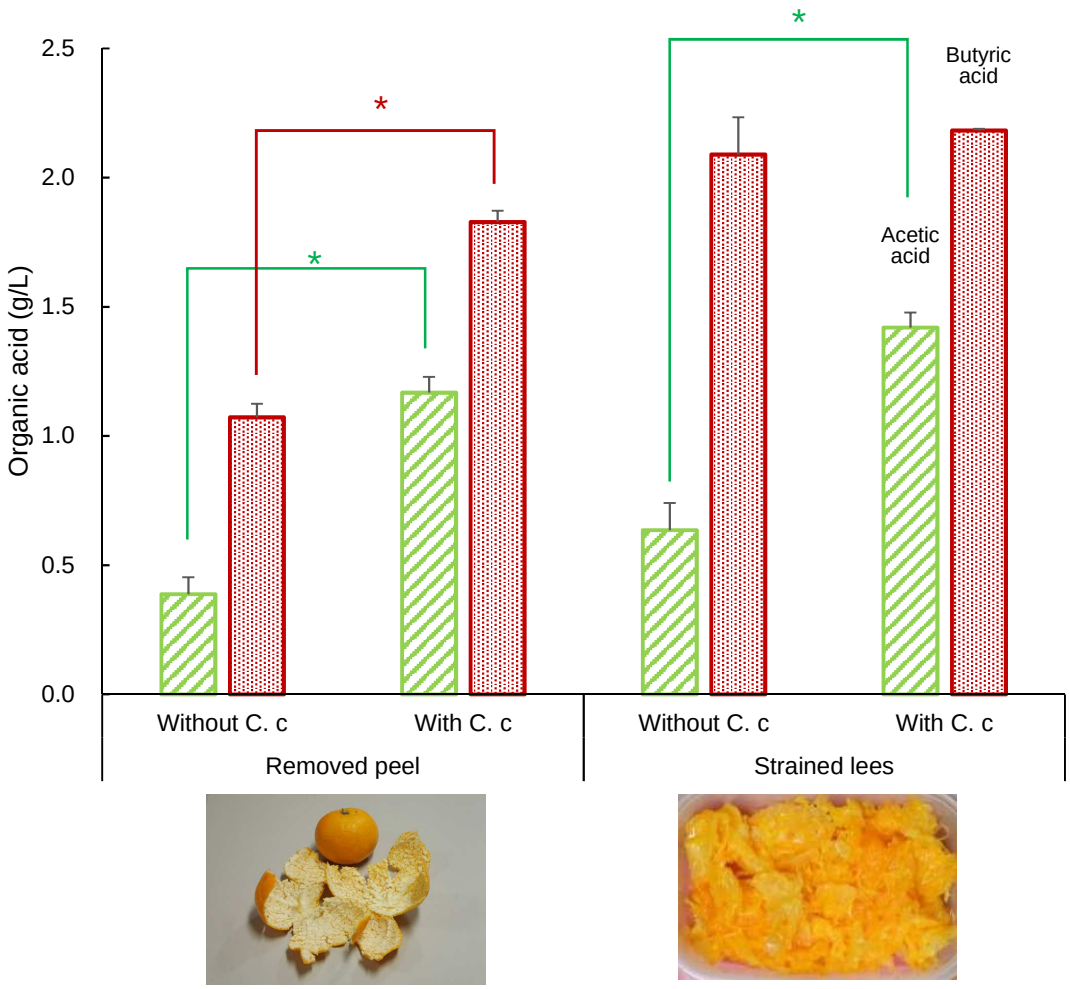


Figure 12. Organic acid concentration in the culture supernatant from removed peel and strained lees with or without *C. cellulovorans*. Hatched bars and dotted bars indicate Acetic acid and Butyric acid, respectively. Values are means ± SE of five independent samples. An asterisk indicates a significant difference ($p < 0.05$).

3.1.6. Consolidated bioprocessing of *C. cellulovorans* and *C. beijerinckii*

C. cellulovorans, which was precultured anaerobically in the media with 0.5% (w/v) Avicel at 37 °C without shaking for 4 days, was used. *C. beijerinckii*, which was precultured anaerobically with glucose for 1 day, was used. Dry weight of the removed peel was calculated from the water content, which was 71.6%. The removed peel, which was 0.28 g of wet weight, was added into *C. cellulovorans* medium as 2.5% (w/v) of a dried substrate. The final volume of the medium as approximately 8 ml for each vial. 12 vials were made. *C. cellulovorans* was inoculated in 3 vials, *C. cellulovorans* and *C. beijerinckii* were inoculated at the same time in other 3 vials which were consolidated bioprocessing (CBP), *C. beijerinckii* was inoculated in other 3 vials and noting was inoculated in the remaining 3 vials which were negative control. Inoculation volumes of *C. cellulovorans* and *C. beijerinckii* were 1.5 ml and 0.15 ml, respectively. Removed peel in the media that were inoculated with *C. cellulovorans* alone and CBP were almost completely degraded for 72 h cultivation (**Figure 13**). Furthermore, total sugar concentration was reduced 96% in both *C. cellulovorans* alone and CBP (**Figure 14a**). It was clearly demonstrated that CBP was able to degrade removed peel as the same as *C. cellulovorans* alone in spite of the higher concentration of the substrate, 2.5 %. And also, the volume of the substrate after degradation was much less than that in the negative control and was able to be easily separated by a centrifugation. Furthermore, it was clearly revealed that *C. beijerinckii* was able to grow in the removed peel culture including limonene because of the higher butyric acid concentration and gas production compared with that in the negative control.

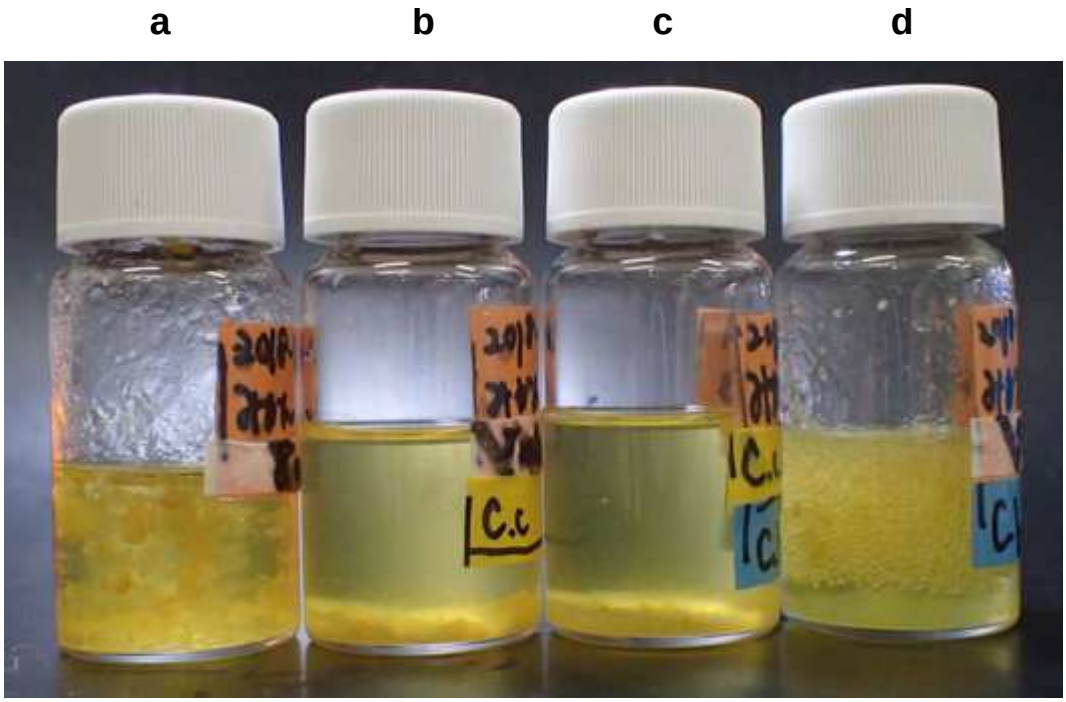


Figure 13. Nothing was inoculated (Negative control) (**a**), *C. cellulovorans* was inoculated (**b**), *C. cellulovorans* and *C. beijerinckii* were inoculated (CBP) (**c**), and *C. beijerinckii* was inoculated (**d**) after 72 h cultivation.

However, butanol was not detected in any cultures. Only ethanol was detected and there was no significant difference (**Figure 14b**). On the other hand, reducing sugar concentration in the culture inoculated Clostridia reduced significantly compared with that of negative control (**Figure 14c**). Interestingly, reducing sugar concentrations in the culture inoculated *C. beijerinckii* were lower than the culture inoculated with *C. cellulovorans* alone. It was suggested that there were some saccharides that *C. beijerinckii* preferred in the medium and *C. beijerinckii* consumed these saccharides even when *C. cellulovorans* existed together. Therefore, the possibility was revealed that cellulosic biomass degradation by *C. cellulovorans* and the fermentation by *C. beijerinckii* carry out simultaneously, being exactly CBP. Furthermore, concentrations of formic acid and acetic acid increased but butyric acid concentration did not increase in the culture inoculated *C. cellulovorans*. On the other hand, concentrations of acetic acid and butyric acid increased in the culture of CBP. Lactic acid concentration increased in the culture inoculated *C. cellulovorans* alone, and no formic acid and acetic acid increased in the culture inoculated *C. beijerinckii* alone, and also butyric acid concentration in CBP was the highest (**Figure 15**). This result also suggested that *C. cellulovorans* and *C. beijerinckii* grew together producing formic acid by *C. cellulovorans* and butyric acid by *C. beijerinckii* and *C. cellulovorans*.

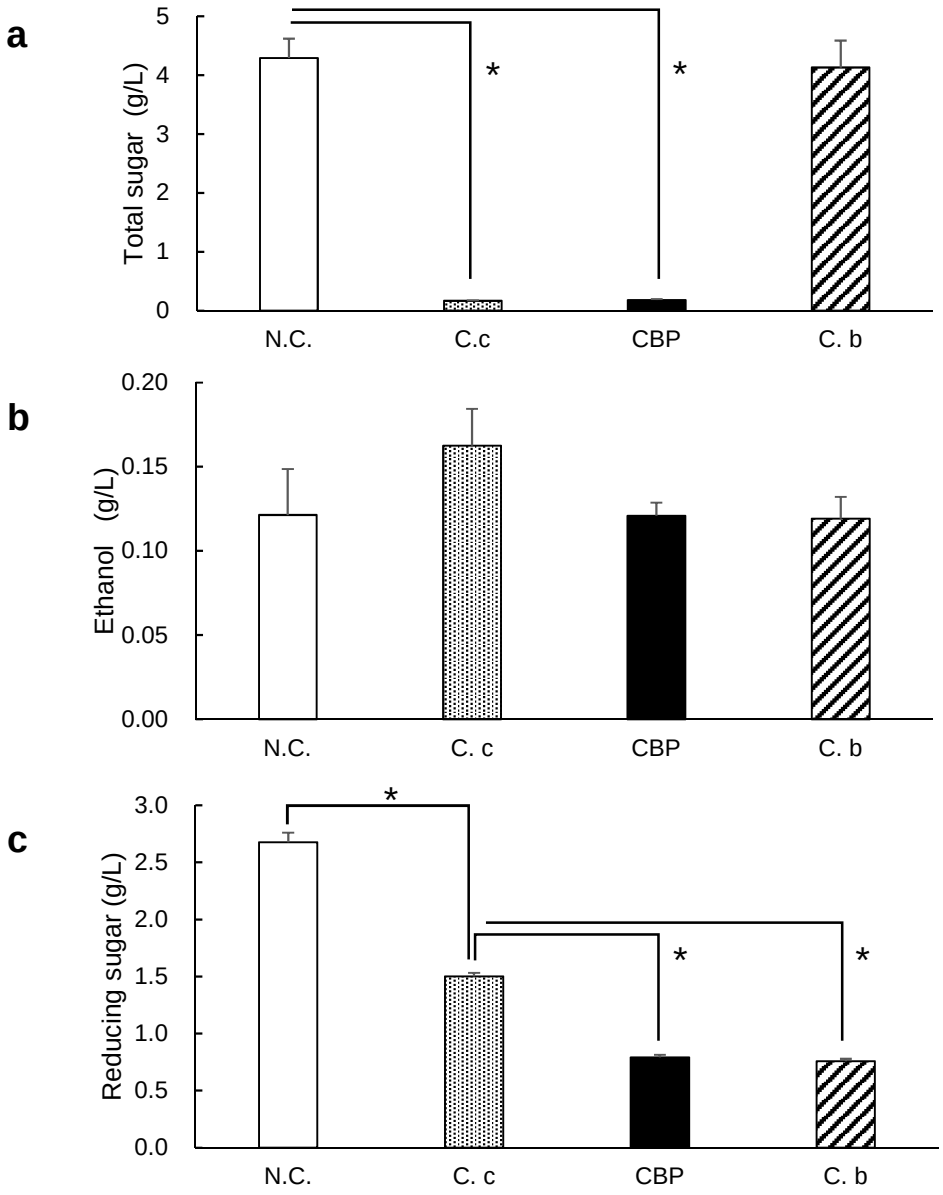


Figure 14. (**a**) Total sugar concentration in the cultures, where negative control (open bar), *C. cellulovorans* alone (dotted bar), CBP (closed bar), *C. beijerinckii* alone (hatched bar) are included. (**b**) Ethanol concentration in the culture, where negative control (open bar), *C. cellulovorans* alone (dotted bar), CBP (closed bar), *C. beijerinckii* alone (hatched bar) are included. (**c**) Reducing sugar concentration in the culture supernatant, where negative control (open bar), *C. cellulovorans* alone (dotted bar), CBP (closed bar), *C. beijerinckii* alone (hatched bar) are included. Values are means ± SE of five independent samples. An asterisk indicates a significant difference ($p < 0.05$).

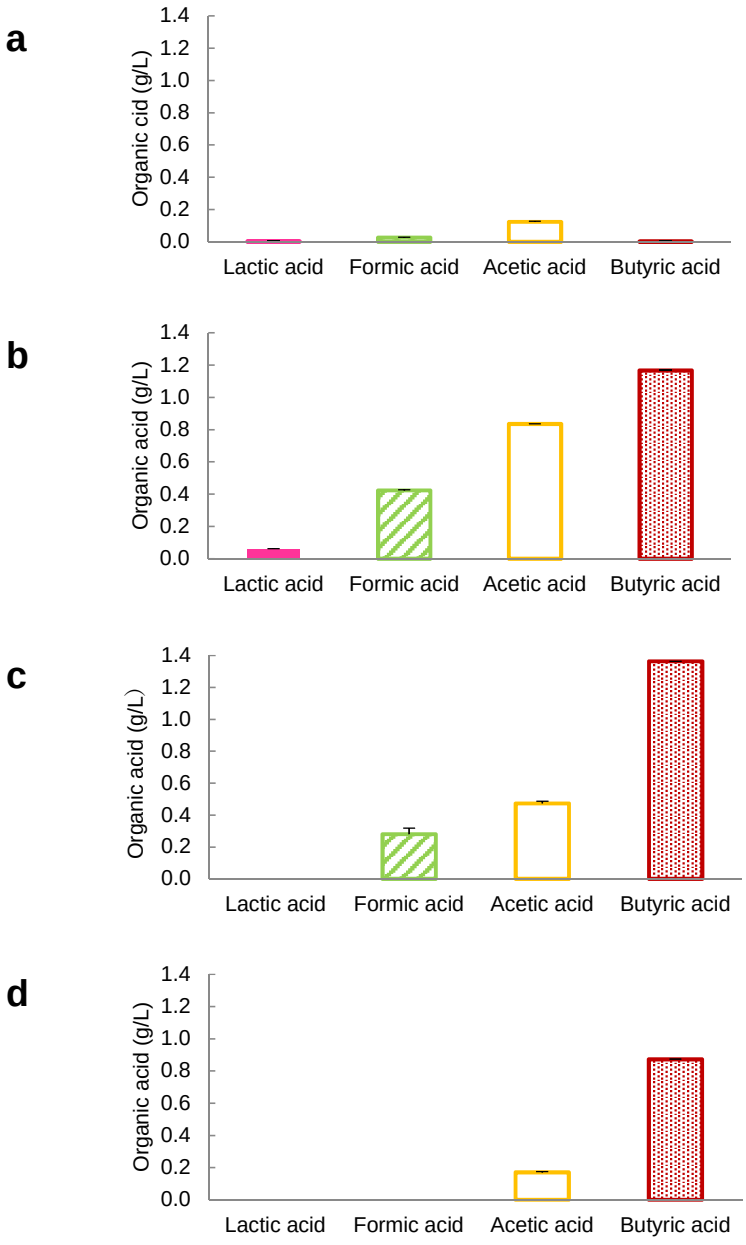


Figure 15. Organic acid concentration in the culture supernatant, where (**a**) negative control, (**b**) *C. cellulovorans* alone, (**c**) CBP, (**d**) *C. beijerinckii* alone are showed. Lactic acid (close bar), formic acid (hatched bar), acetic acid (open bar) and butyric acid (dotted bar) are indicated. Values are means ± SE of five independent samples. An asterisk indicates a significant difference ($p < 0.05$).

3.2.1. 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 (**Figure 16**). Next, Avicel was used for a reference of cellulose degradation with *C. cellulovorans*. The inoculation volume with a *C. cellulovorans* monoculture was decided, according to measured cell growth on the precultures. As a result, the initial RLU value of the monoculture closely reached 1,000, 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 concentrations of total sugar, reducing sugar and organic acid, cell growth and gas production were measured for 11-days cultivations. *C. cellulovorans* degraded 87.3% SBP and 86.3% Avicel, respectively, without any pretreatment (**Figure 17a**). Interestingly, the maximum cell growth in the Avicel culture was 5-days after inoculation, which was the second peak in the profile, while that in the SBP culture was 1-day after inoculation, which was the first peak in the profile, but both SBP and Avicel culture had the first peak and the second peak (**Figure 17b**). On the other hand, reducing sugar concentration at 0-day in SBP culture was more than two times higher than that in Avicel culture (**Figure 17c**). The difference of the first peaks suggested that while *C. cellulovorans* grew rapidly utilizing rich reducing sugar and fresh mineral in SBP culture, *C. cellulovorans* grew slowly due to less reducing sugar in Avicel culture. Furthermore, because gas production in Avicel culture became active a couple of days after inoculation, it was suggested that *C. cellulovorans* needed approximately a couple of days to prepare cellulosome and non-cellulosomal enzymes, and then the degradation became active after a few days. The second peak in Avicel

culture was more than six times higher than that in SBP culture. This result suggested that Avicel, which had higher total sugar concentration than SBP, was degraded and cellobiose was released, which then allowed *C. cellulovorans* to grow thrivingly utilizing rich cellobiose. More interestingly, butyric acid concentration in Avicel started to increase simultaneously with cell growth, however butyric acid concentration in SBP culture hardly increased (**Figure 18a**). Butyric acid concentrations of Avicel and SBP culture were significantly different (**Figure 18b**). On the other hand, concentrations of formic acid and acetic acid in Avicel and SBP culture were not significantly different, and formic acid and acetic acid were major products in SBP culture. It was suggested that a metabolic pathway in *C. cellulovorans* might be different between the SBP and Avicel cultures (**Figure 19**). According to the gas production in the SBP and Avicel cultures, H_2 productions were 28.6 liter per 1 kg of dried SBP and 132 liter per 1 kg of Avicel, respectively (**Figure 18c**). Therefore, the decrease of the total sugar in the SBP culture seems reasonable to produce 28.6 liter of H_2 whose concentration was close to 22% of 132 liter of H_2 in the Avicel culture. Thus, it indicated that *C. cellulovorans* degraded cellulosic biomass to produce H_2 which should be a raw material of CH_4 by the CO_2 reduction pathway in methanogens.

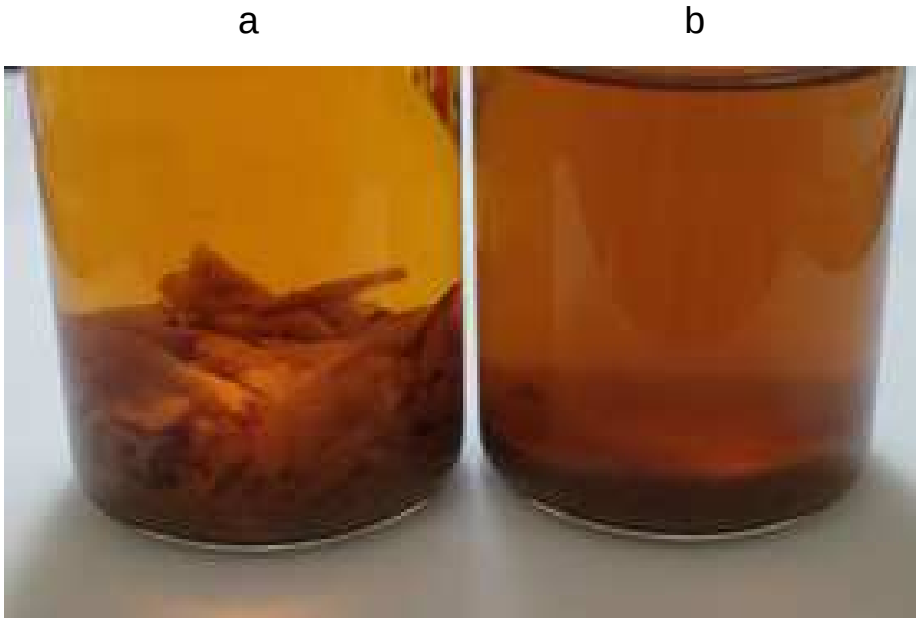


Figure 16. The cultures after the cultivation of *C. cellulovorans* with SBP. (**a**) Negative control. (**b**) The cultivation of *C. cellulovorans*. SBP used in the culture media was not pretreated by milling.

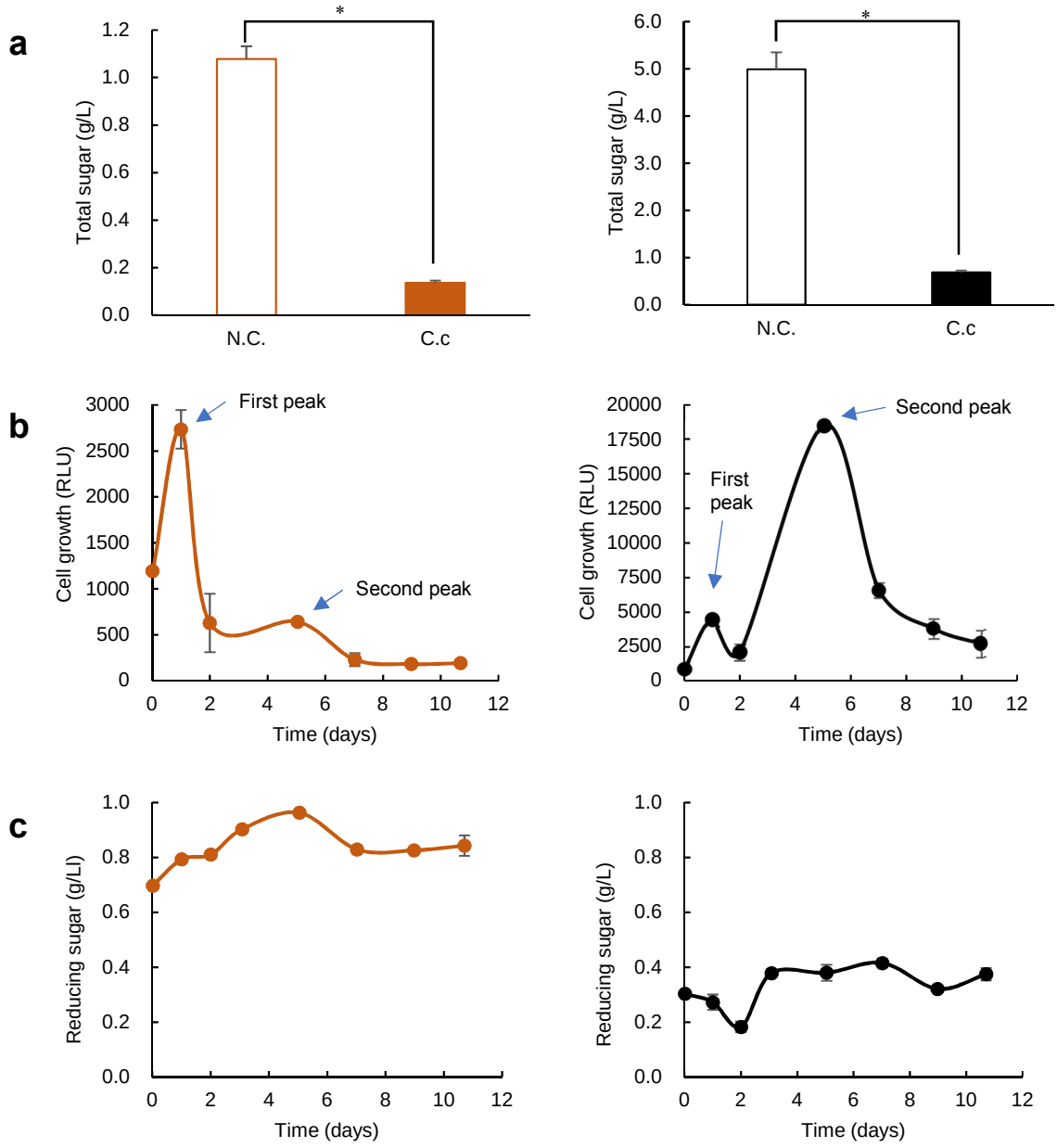


Figure 17. Cultivation of *C. cellulovorans* with SBP and Avicel. (**a**) Total sugar concentration after 11-days cultivation in the culture with SBP (left) and Avicel (right), where negative control (open bar), *C. cellulovorans* (closed bar) are included. (**b**) Cell growth in the culture with SBP (left) and Avicel (right). (**c**) Reducing sugar concentration in the culture with SBP (left) and Avicel (right). Values with error bars are mean ± SE of three independent samples. An asterisk indicates a significant difference ($p < 0.05$).

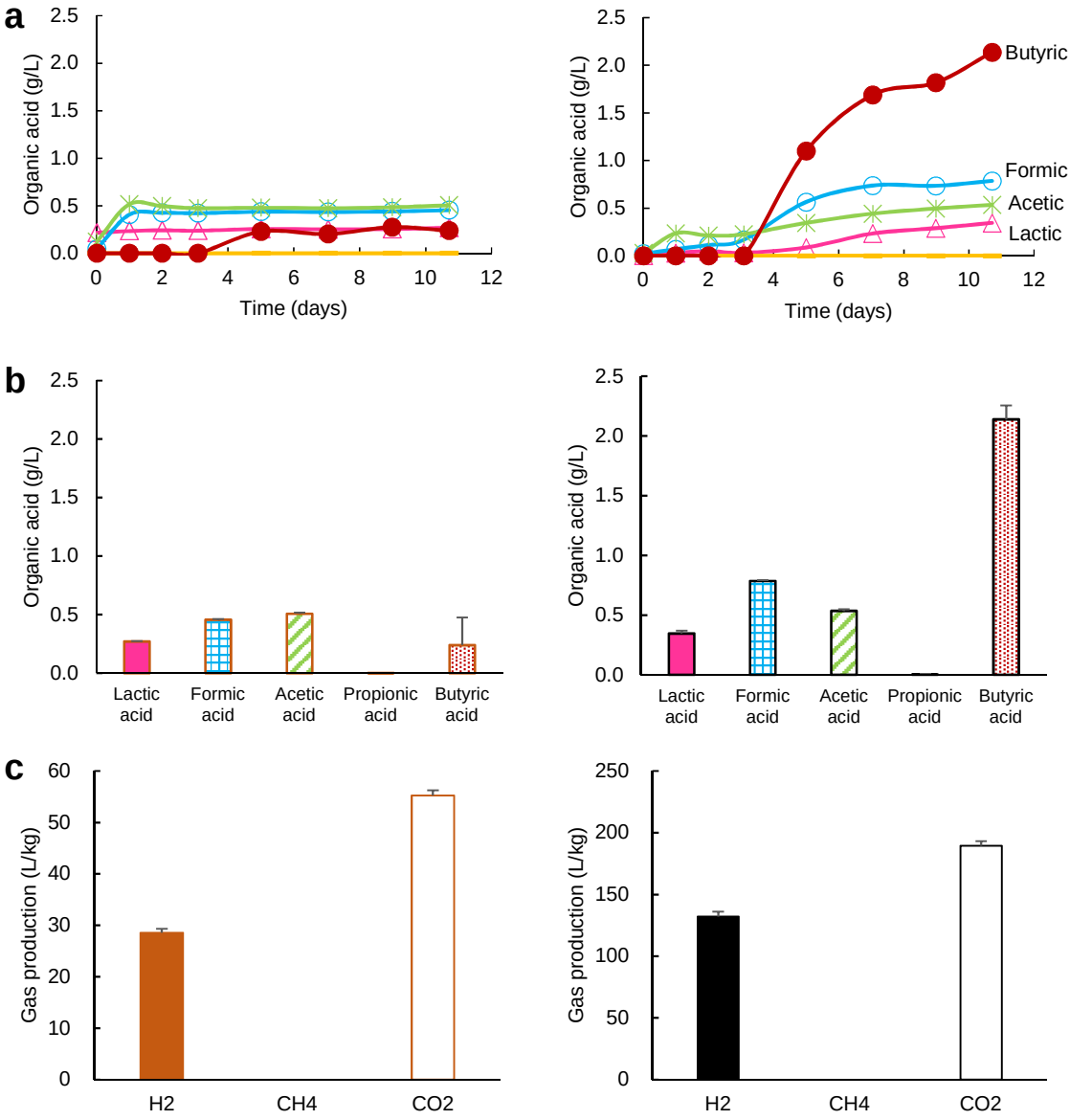


Figure 18. Cultivation of *C. cellulovorans* with SBP and Avicel. (**a**) Organic acid concentration in the culture with SBP (left) and Avicel (right), where lactic acid (Δ), acetic acid (*), butyric acid (filled circle), formic acid (open circle) and propionic acid (flat bar) are included. (**b**) Organic acid concentration in the culture with SBP (left) and Avicel (right) after 11-days cultivation. (**c**) Gas production after 11-days cultivation in the culture with SBP (left) and Avicel (right), where H_2 (closed bar), CH_4 (hatched bar), CO_2 (open bar) are included. Values with error bars are mean ± SE of three independent samples.

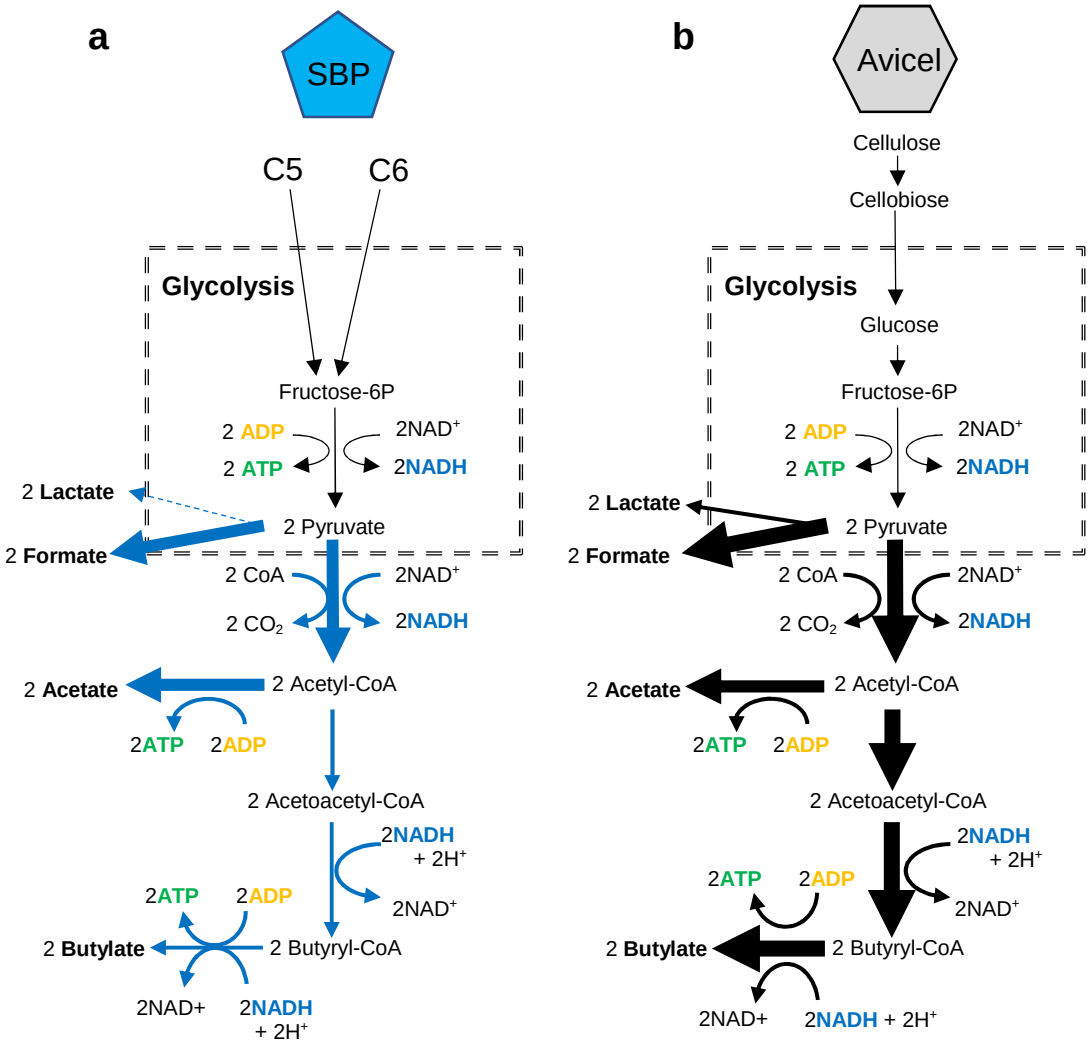


Figure 19. Metabolic pathway of organic acid production in *C. cellulovorans* with (**a**) SBP and (**b**) Avicel as a substrate, modified **Figure 2**.

3.2.2. All- inclusive analysis of microbial flora including Methanogen

Based on the 16S rRNA sequencing, a total of 2,359 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 *Methanosaetaceae*, *Methanosaeta*, and *Methanospirillaceae* were also identified. More interestingly, the genus *Methanosaeta*, which utilizes only acetic acid, was a large portion of ratio next to *Methanosarcina* (**Table 3**). Dominant families were identified and found to be belonging to *Syntrophomonadaceae* (11.37%), *Marinilabiaceae* (5.59%), *Clostridiaceae* (4.91%), and *Spirochaetaceae* (4.52%) (**Figure 20**).

Table 2. Identified 17 class and their species by 16S rRNA sequencing.

Kingdom	Phylum	Class	Order	Family	Genus	Species	
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*	*mazei*	1.340%
Bacteria	*Thermotogae*	*Thermotogae*	*Thermotogales*	*Thermotogaceae*	*Kosmotoga*	*mrcj*	0.278%
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Syntrophomonadaceae*	*Syntrophomonas*	*wolfei*	0.099%
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Desulfosporosinus*	*meridiei*	0.073%
Bacteria	*Fibrobacteres*	*Fibrobacteria*	*Fibrobacterales*	*Fibrobacteraceae*	*Fibrobacter*	*succinogenes*	0.039%
Bacteria	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Arcobacter*	*cryaerophilus*	0.009%
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	*butyricum*	0.005%
Bacteria	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	*Actinomyces*	*europaeus*	0.002%
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*lwoffii*	0.002%
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	*ovatus*	0.002%
Bacteria	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*flexus*	0.001%
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*	*fortis*	0.001%
Bacteria	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	*manihotivorans*	0.001%
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Serratia*	*marcescens*	0.001%
Bacteria	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	*ruber*	0.001%
Bacteria	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	*succinicans*	0.001%
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	*sulfonivorans*	0.001%

Table 3. Identification of methanogens by 16S rRNA sequencing.

Kingdom	Phylum	Class	Order	Family	Genus	Species	
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*		1.34%
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosaetaceae*	*Methanosaeta*		0.54%
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanospirillaceae*			0.25%
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanoculleus*		0.22%
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanocorpusculaceae*	*Methanocorpusculum*		0.09%
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*		0.09%
Archaea	*Euryarchaeota*	*Methanomicrobia*	*Methanomicrobiales*	*Methanomicrobiaceae*	*Methanofollis*		0.07%
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*		0.04%
Archaea	*Euryarchaeota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*		0.02%
Archaea	*miscellaneous*						0.25%

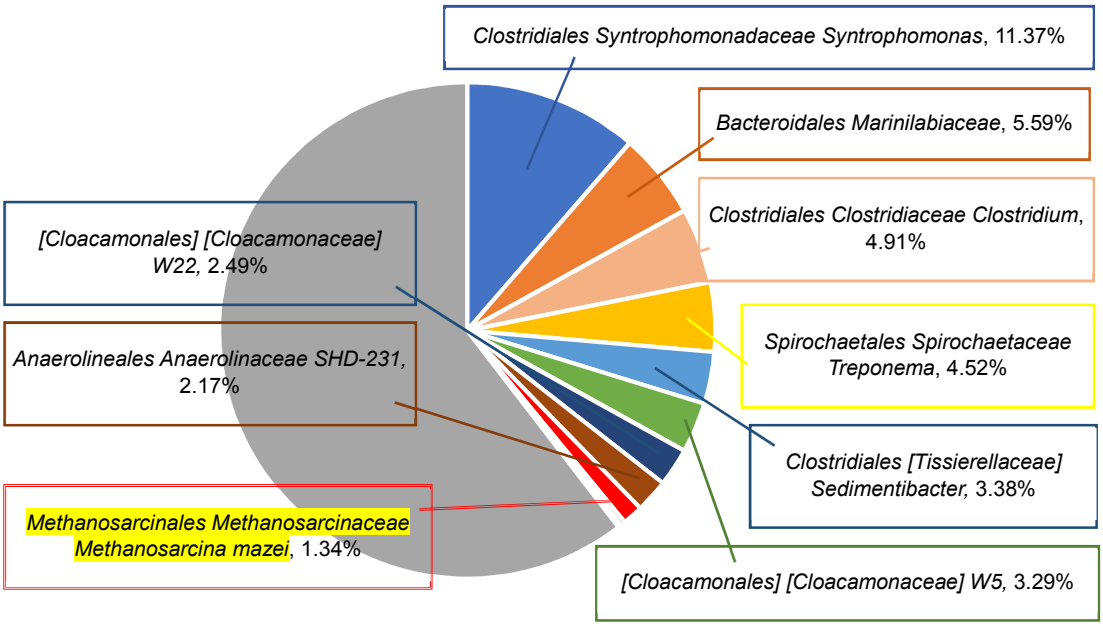


Figure 20. Dominant families in MFMP by 16S rRNA sequencing.

3.2.3. Precultivation of *C. cellulovorans* and MFMP

The inoculation volume to the MFMP monoculture was decided as the same as the *C. cellulovorans* monoculture, so that the initial RLU values of each monoculture closely reached to 1,000. The RLU value of the MFMP preculture with 0.5% (w/v) glucose and 0.25% (w/v) cellobiose 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 1,036. 2 ml of the *C. cellulovorans* preculture and 3 ml of the MFMP preculture, respectively, were inoculated in the CCeM culture, so that the cell growth: substrate ratio became the same as monocultures.

3.2.4. 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 SBP at 37 °C without shaking. The total sugar of the MFMP culture hardly decreased. However, surprisingly, the total sugar of the CCeM culture decreased by 86.0%, which was not significantly different compared to *C. cellulovorans* monoculture (**Figure 21a**). Furthermore, cell growth of the CCeM culture was higher than that of the MFMP culture during 2- 6 days cultivation and the profile of the CCeM culture had two peaks as with the RLU profile of the *C. cellulovorans* monoculture (**Figure 21b**). On the other hand, the reducing sugar concentrations decreased from the initial value in the CCeM and MFMP cultures (**Figure 21d**). Interestingly, pH in the MFMP culture was maintained to be approximately 7, while pH in the CCeM was 6.57, which was lower than pH in the *C. cellulovorans* culture, 6.82 (**Figure 21c**).

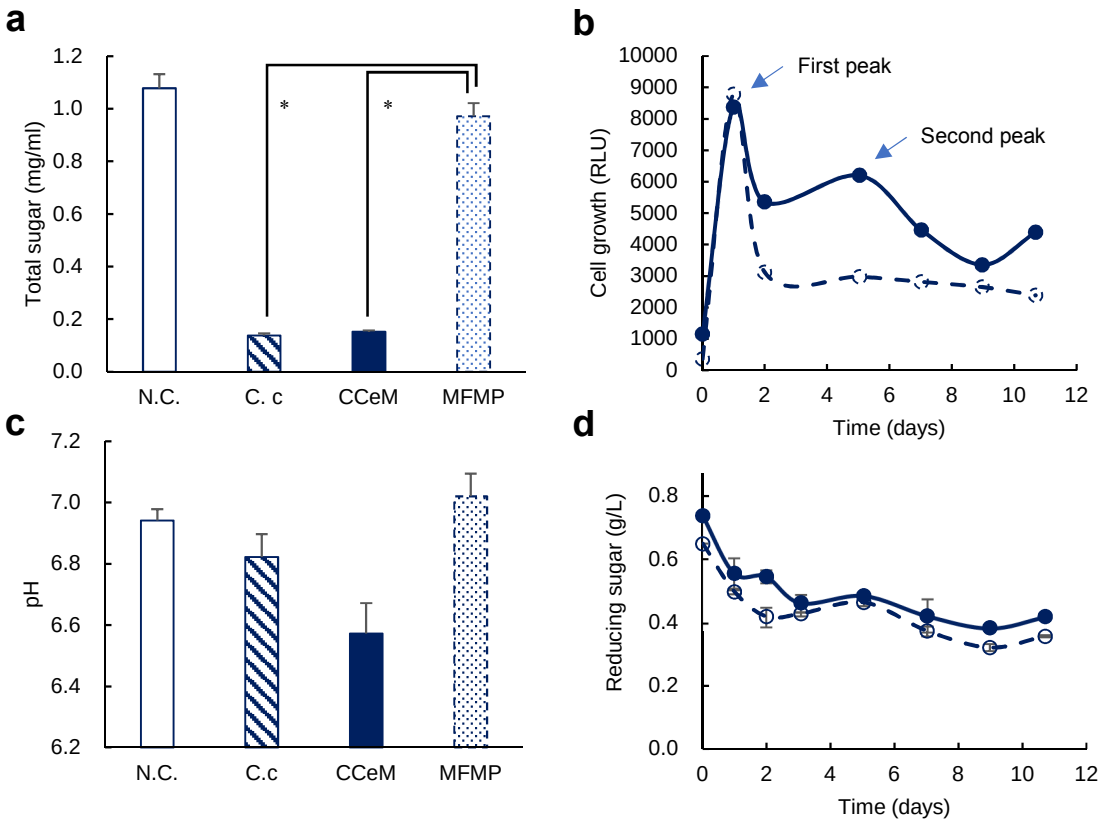


Fig. 21 Cultivation of *C. cellulovorans*, CCeM and MFMP with SBP. (**a**) Total sugar concentration after 11-days cultivation in the culture with SBP, where negative control (open bar), *C. cellulovorans* (hatched bar), CCeM (closed bar), MFMP (dotted bar) are included. (**b**) Cell growth in the culture of CCeM and MFMP with SBP, where CCeM (○_open circle), MFMP (●_closed circle). (**c**) pH in the cultivation with SBP, where negative control (open bar), *C. cellulovorans* (hatched bar), CCeM (closed bar), MFMP (dotted bar) are included. (**d**) Reducing sugar concentration of 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 ($p < 0.05$).

Regarding with gas production, CO_2 production in CCeM and MFMP cultures were two times higher than that in the *C. cellulovorans* monoculture (**Figure 22a**). It suggested that various microbes in the MFMP consumed the reducing sugar and produced CO_2 the CCeM and MFMP cultures. Thus, it was demonstrated that *C. cellulovorans* was able to coexist with methanogens and various other microbes and to degrade SBP, while the degradation performance of *C. cellulovorans* was maintained. For biogas production, 34.0 L/kg of CH_4 and 110 L/kg of CO_2 were measured in the CCeM culture, respectively. On the other hand, 48.2 L/kg of CH_4 and 105 L/kg of CO_2 in the MFMP culture were measured, respectively. It was also revealed that MFMP was able to produce CH_4 coexisting with *C. cellulovorans*. More interestingly, H_2 was not accumulated in both cultures, and the final volume of H_2 was less than that in negative control, although 28.6 L/kg H_2 was produced in the *C. cellulovorans* monoculture. These results suggested that methanogenic bacteria, such as *M. mazei*, generated CH_4 from H_2 and CO_2 by the CO_2 reduction pathway. From the perspective of organic acid, which wes other metabolic products except gas, the increase of acetic acid concentration was characteristic (**Figure 22b**). Interestingly, propionic acid concentration increased, which did not accumulate in the culture with *C. cellulovorans* alone. However, concentrations of formic acid and lactic acid did not increase.

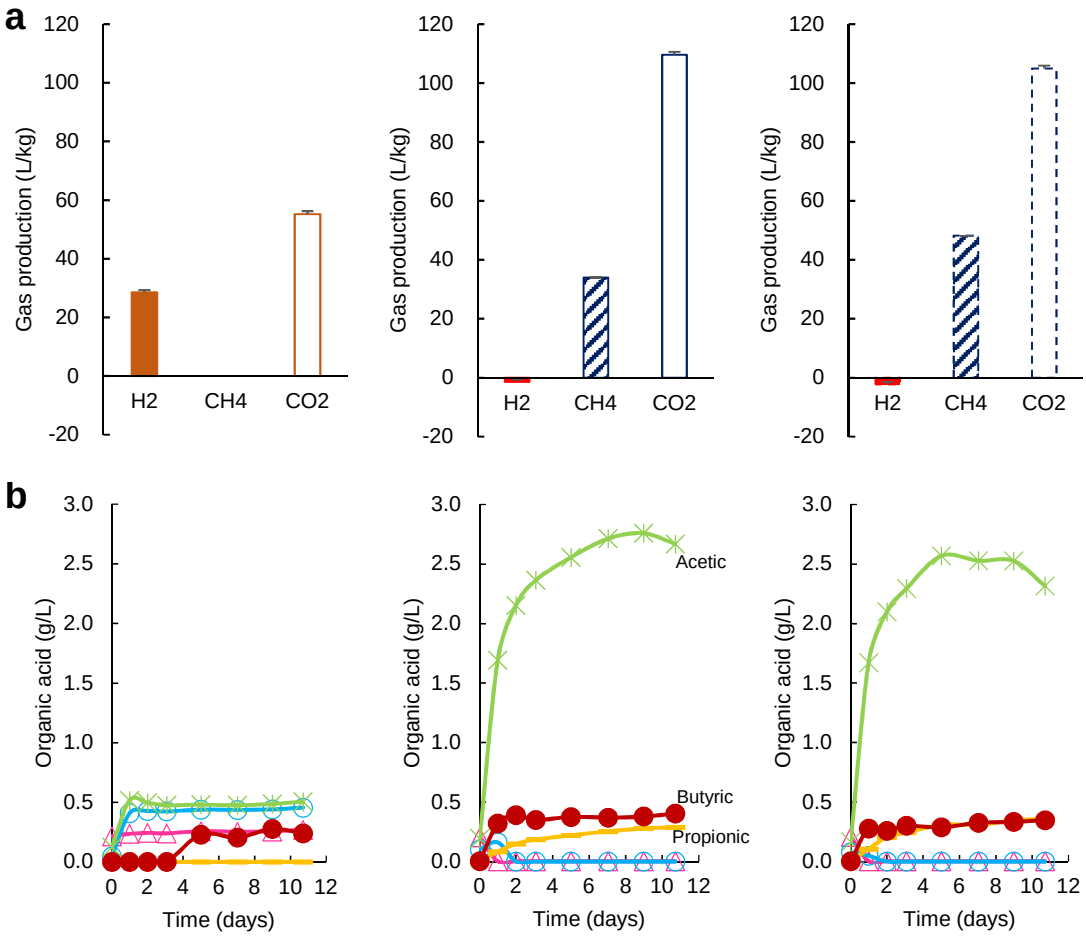


Fig. 22 Cultivation of *C. cellulovorans*, CCeM and MFMP with SBP. (**a**) Gas production after 11-days cultivation in the *C. cellulovorans* (left), CCeM (center) and MFMP (right) cultures with SBP, where H_2 (closed bar), CH_4 (hatched bar), CO_2 (open bar) are included. Values indicate increments from the volume of negative control and are calculated as the volume per one kg of dry weight of SBP. (**b**) Organic acid concentration in the *C. cellulovorans* (left), CCeM (center) and MFMP (right) cultures with SBP, where lactic acid (Δ), acetic acid (*), butyric acid (filled circle), formic acid (open circle) and propionic acid (flat bar) are included. Values with error bars are mean ± SE of three independent samples.

3.2.5. Degradation and fermentation with orange wastes and sorghum by CCeM and MFMP

Anaerobic batch cultivations of *C. cellulovorans*, CCeM and MFMP were carried out with removed peel, strained lees and sorghum as a substrate containing 0.5% (w/v) of each of them at 37 °C without shaking. As unexpected, total sugar concentration in the CCeM culture hardly decreased (**Figure 23, left**). Sorghum was the most difficult to degrade in this paper, there was no second peak on the cell growth profile in the *C. cellulovorans* culture (**Figure 23a, center**). On the other hand, H_2 accumulated in the CCeM culture with sorghum, removed peel and strained lees, and it was suggested that CO_2 reduction pathway did not work well in the CCeM culture (**Figure 23, right**). This result demonstrated that degradation and fermentation of CCeM depended on the type of the substrate.

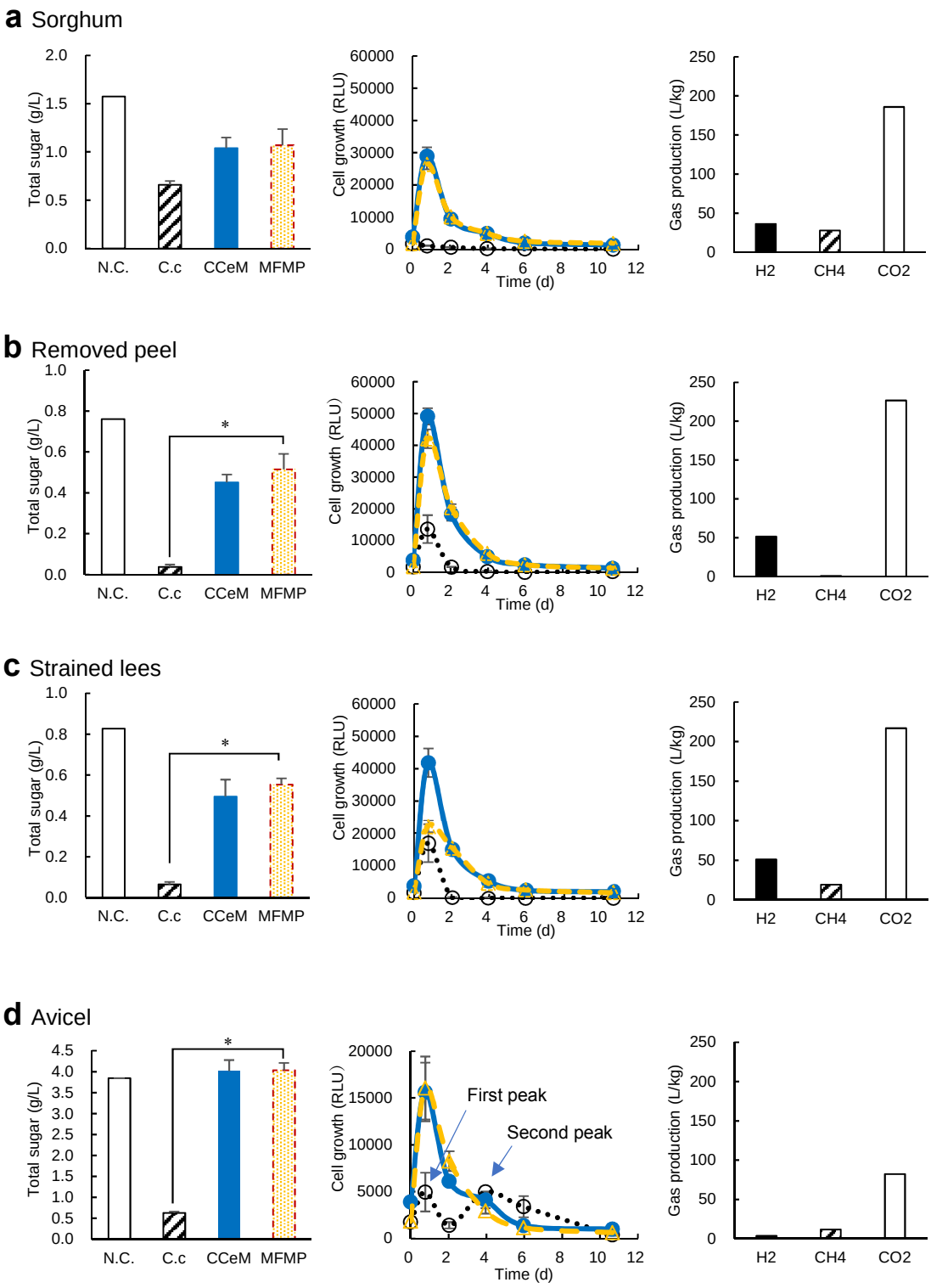

a Sorghum
Total sugar (g/L)
2.0
1.5
1.0
0.5
0.0
N.C.
C.c
CCeM
MFMP
Cell growth (RLU)
60000
50000
40000
30000
20000
10000
0
0 2 4 6 8 10 12
Time (d)
Gas production (L/kg)
250
200
150
100
50
0
H2
CH4
CO2
b Removed peel
Total sugar (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
*
N.C.
C.c
CCeM
MFMP
Cell growth (RLU)
60000
50000
40000
30000
20000
10000
0
0 2 4 6 8 10 12
Time (d)
Gas production (L/kg)
250
200
150
100
50
0
H2
CH4
CO2
c Strained lees
Total sugar (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
*
N.C.
C.c
CCeM
MFMP
Cell growth (RLU)
60000
50000
40000
30000
20000
10000
0
0 2 4 6 8 10 12
Time (d)
Gas production (L/kg)
250
200
150
100
50
0
H2
CH4
CO2
d Avicel
Total sugar (g/L)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
N.C.
C.c
CCeM
MFMP
Cell growth (RLU)
20000
15000
10000
5000
0
First peak
Second peak
0 2 4 6 8 10 12
Time (d)
Gas production (L/kg)
250
200
150
100
50
0
H2
CH4
CO2

Figure 23. Cultivation of *C. cellulovorans*, CCeM and MFMP with sorghum (**a**), removed peel (**b**), strained lees (**c**) and Avicel (**d**). For **a**, **b**, **c** and **d**, total sugar concentration (left), cell growth (center), where *C. cellulovorans* (○_open circle), CCeM (Δ_open triangle) and MFMP (●_closed circle) are included, and gas production (right). Values with error bars are mean ± SE of three independent samples. An asterisk indicates a significant difference ($p < 0.05$).

4. Discussion

Although the purchase price of cellulosic feedstocks is competitive with petroleum on an energy basis, the cost of lignocellulose conversion to biofuels using today's technology is high [103]. Furthermore, cost reductions can be pursued via either in-paradigm or new-paradigm innovations. In this study, since both *C. beijerinckii* and *C. cellulovorans* are mesophilic anaerobes and grown at 37 °C, it was assumed that consolidated bioprocessing (CBP) between them was synergistically carried out in the same media. It has been reported that *C. cellulovorans* was able to degrade not only cellulose but also corn fibers and plant cell walls such as cultured tobacco and Arabidopsis thaliana by formation of their protoplasts [43,58]. Therefore, mandarin orange wastes hit upon a good target for direct IBE fermentation by *C. beijerinckii*. First, the high tolerance of *C. beijerinckii* and *C. cellulovorans* against limonene toxicity was demonstrated. Whereas both *C. beijerinckii* and *C. cellulovorans* showed ethanol production when cultivated in even 0.05% limonene, *S. cerevisiae* revealed no production of ethanol in 0.05% limonene (**Figure 5a**). In general, it is said that a mandarin orange includes 0.01–0.2% limonene based on season and orange species. In the case of *C. cellulovorans*, it degraded 93% of removed peel and 85% of strained lees, respectively (**Figure 8, 9**). On the other hand, *C. beijerinckii* produced 0.046 g of butanol per 1 g of strained lees as a dried weight in the culture supernatant with *C. cellulovorans* (**Figure 10**). According to several butanol yields that have been reported in IBE or ABE fermentation by *C. beijerinckii*, butanol (g) per 1 g of glucose was the range within 0.17 to 0.22 g/g [75,76,104]. The reducing sugar concentration in the supernatant of strained lees before *C. beijerinckii* inoculation was 1.68 g/L and butanol concentration from the supernatant was approximately 0.28 g/L. The calculated butanol yield is 0.17 g/g and is reasonable compared with the previous reports. These results indicated that there were great

advantages to the combination of saccharification and IBE fermentation by mesophilic *C. cellulovorans* and *C. beijerinckii*. Furthermore, *C. cellulovorans* does not require any pretreatment machines, tools or chemicals to degrade mandarin orange wastes. However, this study showed butanol yields by *C. beijerinckii* were different depending on the type of substrates, such as removed peel and strained lees of the mandarin orange. Detailed analyses of sugar utilization and its metabolite pathways of *C. beijerinckii* could be feasible and is necessary for more studies. Also, it could be easier to optimize the butanol yields by *C. beijerinckii* monoculture rather than the co-culture system of *C. cellulovorans* and *C. beijerinckii*. Under the culture conditions optimized for *C. cellulovorans*, orange wastes were quickly degraded and the volume was reduced, suggesting that it could be easily recovered by centrifugation (**Figure 13**). Furthermore, the individual culture broth would be used as bacterial source in the next degradation batch. Likewise, after the centrifugation, the culture supernatant can be optimized for *C. beijerinckii* and the culture broth will be inoculated to the next fermentation batch by cell recycling. In the co-culture system of *C. cellulovorans* and *C. beijerinckii* in a tank, the degradation of orange wastes and fermentation were also performed (**Figure 14c, 15b**), however fermented products varied. It might be necessary to optimize the inoculation ratio of both, but it is difficult to adjust the ratio to inoculate into the next treatment batch from the co-culture broth. Even if it is not the co-culture, it is possible to construct the consolidated process utilizing *C. cellulovorans* and *C. beijerinckii* both without extra enzymes degrading cellulosic biomass. Thus, by degrading orange wastes, the volume of waste will be reduced, decreasing the costs of drying and transporting such wastes [71]. In fact, water contents of removed peel and strained lees were 71.6% and 83.9%, respectively. Furthermore, by consolidated bioprocessing from orange wastes, biobutanol will take the place of fossil fuels such as gasoline and will save energy on the current

process. Finally, it was a surprising result that the cell growth rapidly decreased immediately after inoculation reaching the value equivalent to that of negative control in the medium containing limonene, and that the cell growth turned to grow again and Avicel was degraded (**Figure 7c, d**). From the fact that re-proliferation was observed, it is unlikely that all cells were killed by the presence of limonene, and there is a possibility that cells turned to spores rapidly under the stimulus of the presence of limonene. Therefore, the result provides a way to explore the detail of the sigma factor by examining gene expression in the case of adding limonene [105].

The biomethanation process is not a single process. Three anaerobic microbes such as fermentative microbes, acetogenic microbes and methanogens mainly participate in the methanation [106,107,108]. In fact, methanogens require acetate, H_2 and CO_2, which are precursors for methanogenesis, to metabolize CH_4 by two major pathways such as the acetoclastic pathway and the CO_2 reduction pathway [109]. 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 [62], where *C. cellulovorans* produced H_2, acetate, butyrate, and lactate as the obligatory fermentation products from cellulose degradation, and *M. barkeri* was able to further utilize H_2, formate, and acetate for methanogenesis by both the CO_2 reduction and acetoclastic pathways. In this study, we demonstrated that the CCeM was able to degrade SBP and produce CH_4 simultaneously in a single tank. In fact, SBP included highly suitable substrates for bioconversion by the CCeM. Although *C. cellulovorans* was able to grow on the medium containing 0.5% cellobiose, some bacteria can never utilize it. In fact, after cultivation of *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, *C. cellulovorans* degraded SBP to produce a variety of saccharides which could be utilized by various microbes in MFMP. Using SBP in such processes would be a great benefit in reducing the cost of drying and transporting SBP in sugar factories. Exoproteome analysis of *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 [110]. More interestingly, in comparison of the co-cultures between *C. cellulovorans–M. barkeri* and *C. cellulovorans–M. mazei*, the pattern of gene expression on a cellulose encoding Clocel_0905 was completely different from the combination between *M. barkeri* and *M. mazei* [62]. This result indicated that it might have another possibility of cellulose degradation manners via microbial interactions. In this study, the butyric acid concentration in SBP culture did not increase much, although that of formic acid and acetic acid immediately increased after 1-day of cultivation (**Figure 18a- left**). This suggests that *C. cellulovorans* grew and produced butyric acid later, because the starting point of its growth was delayed until cellulosome and non-cellulosomal enzymes were secreted and accordingly started to degrade Avicel (**Figure 18a-right**). As previous papers stated, changes in cellulosome occurs in the presence of sugars other than glucose [47], and faster growth is achieved in media containing xylan and cellulose by assimilating xylan first [111], it was suggested that a metabolic pathway seems to be different between the SBP and Avicel cultures (**Figure 19**). Shinohara et al. [46] reported fixation of CO_2 in *C. cellulovorans* by the partial operation of the TCA cycle in a reductive manner. In this study, *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 *Clostridium* species. In the genome analysis of *C. cellulovorans* [30], 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 *C. cellulovorans*. In this study, *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 [112]. In addition, gene gain from bacterial taxa is common in at least some Methanosarcina spp. and may often be adaptive [113,114]. 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) [115,116]. Based on the 16S rRNA sequencing, *M. mazei* and the other methanogens were found in MFMP (**Table 2, Figure 20**). In addition, various other miscellaneous microbes also existed. These results revealed that *C. cellulovorans* could survive with MFMP, when their monocultures were mixed so that the initial RLU of *C. cellulovorans* and that of MFMP both reached 1,000. This suggests RLU could be useful as an index when constructing the consortium. In terms of CH_4 yield from SBP, it has been reported that 617 L/kg of CH_4 yields by pretreated SBP, 502.5 L/ kg by using hydrothermal pretreatment and 360 L/kg by adding of external enzymes [87,117,118]. 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 be advantageous on a cost-benefit basis. In addition, since the yield depends on the saccharide 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 for the drying and transporting energy required otherwise (**Figure 16**). Furthermore, 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 (**Figure 21b**), *C. cellulovorans* seems to interact with not only methanogens but also miscellaneous microbes. Therefore, there might be some possibilities that growing miscellaneous microbes in the CCeM increase their RLU and inhibit CH_4 production. It is suggested that there may be a formate- or propionate-mediated interaction between *C. cellulovorans* and methanogens, as lactic acid-mediated interaction has been reported between *Sulfurospirillum multivorans* and *Methanococcus voltae* [119]. Moreover, the accumulation of organic acid in the system means that unused carbons remain. *C. cellulovorans* is a hydrogen-producing bacterium, and by enhancing hydrogen production, it is possible to further convert such unused carbons into methane [120]. On the other hand, the large amount of CO_2 product is not preferable from the viewpoint of reduction of carbon dioxide emission, which is the original purpose, therefore CO_2 which the bacteria itself fixes is important as well as methane production by the CO_2 reduction pathway [121]. Furthermore, formic acid was accumulated in the culture with SBP and *C. cellulovorans*, propionic acid, but not formic acid, was not accumulated in the CCeM and MFMP cultures. This result suggests that formic acid was utilized for hydrogen generation and propionic acid was produced as by-product for methane generation [122,123]. In future studies, it could be possible to find various factors that are not gained from the co-culture between *C. cellulovorans* and methanogens through omics analysis. Furthermore, by using machine learning techniques on such omics data [124], there are some possibilities that it will be able to elucidate not

only inhibit factors for CH_4 production, but also interrelationship between each microbe in the CCeM. This could then be used to improve methane fermentation in the culture with orange wastes and sorghum [125]. Finally, it is interesting to note that CCeM was able to degrade SBP but not Avicel. By examining the details of omics data retrieved at multiple timepoint, the mechanism of why CCeM did not degrade Avicel could potentially be clarified. This would give further insights into improving and maintaining degradation performance of *C. cellulovorans* in a practical use.

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