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Biohydrogen Production from Methane-Derived Biomass of Methanotroph and Microalgae by *Clostridium*

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Abstract: Methane, a potent greenhouse gas, represents both a challenge and an opportunity in the quest for sustainable energy. This work investigates the biotechnology for converting methane into clean, renewable hydrogen. The co-culture of *Chlorella sacchrarophila* FACHB 4 and *Methylomonas* sp. HYX-M1 was demonstrated to completely convert 1 mmol of methane to biomass within 96 h. After acid digestion of such biomass, up to 45.05 µmol of glucose, 4.07 µmol of xylose, and 26.5 µmol of lactic acid were obtained. Both *Clostridium pasteurianum* DSM525 and *Clostridium* sp. BZ-1 can utilize those sugars to produce hydrogen without any additional organic carbon sources. The higher light intensity in methane oxidation co-culture systems resulted in higher hydrogen production, with the BZ-1 strain producing up to 14.00 µmol of hydrogen, 8.19 µmol of lactate, and 6.09 µmol of butyrate from the co-culture biomass obtained at 12,000 lux. The results demonstrate that the co-culture biomass of microalgae and methanotroph has the potential to serve as a feedstock for dark fermentative hydrogen production. Our study highlights the complexities inherent in achieving efficient and complete methane-to-hydrogen conversion, positioning this biological approach as a pivotal yet demanding area of research for combating climate change and propelling the global energy transition.

Keywords: biohydrogen; methane; *Clostridium*; microalgae; methanotrophs

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1. Introduction

Methane (CH_4) is the second major greenhouse gas, and its global warming potential is about 28–34 times higher than that of carbon dioxide, contributing 20% of the global warming effect [1–3]. Methanotrophs are demonstrated to consider methane as the sole carbon and generate carbon dioxide as final product [1,4]. Microalgae are widely distributed in terrestrial and aquatic ecosystems and are capable of fixing carbon dioxide and producing oxygen through photosynthesis [5]. The established co-cultures of aerobic methanotrophs and microalgae show potential for rapid methane utilization without external oxygen supply [6–8]. David successfully established co-cultures of aerobic methanotroph enrichment and microalgae with the supply of methane and carbon dioxide in a continuous reactor [8,9]. These co-cultures not only continuously consumed methane but also accumulated biomass of 641 mg/L [8,9]. Li constructed a co-culture of *Methylocystis*

bryophila and *Scenedesmus obliquus* with biogas feeding. Compared with the single culture of *S. obliquus* of 3×10^7 cells/mL, higher microalgal biomass of 5×10^7 cells/mL was observed [10]. The co-culture of alkaliphilic methanotrophs and *Scenedesmus obtusiusculus* reached a biomass of 3.3 g/L, while alkaliphilic methanotrophs cultured alone only reached a biomass of 0.86 g/L [11]. Furthermore, the co-cultures of *Methylomicrobium alcaliphilum* 20Z and *Chlorella* sp. HS2 led to the final dry cell weight value of 2.4 g/L, whereas the final cell weight values of HS2 and 20Z monocultures were 0.3–0.5 g/L and 0.2–0.3 g/L, respectively [12]. The synergistic cultivation of microalgae with methanotrophic bacteria holds promise in converting methane into biomass; yet, the exploration of subsequent applications for this co-generated biomass remains limited.

Hydrogen is considered as an ideal energy carrier and produces clean combustion with a high-energy density of 122 kJ/g [13,14]. Fermentative hydrogen production from biomass is energy-saving and environmentally friendly, thus providing great potential for industrial application [15,16]. Microalgae and methane-oxidizing bacteria are both high-quality biomass. Some researchers have previously investigated hydrogen production from reducing sugar in microalgal biomass by hydrogen-producing bacteria through batch fermentation [17–19]. For instance, *Clostridium butyricum* CGS5 utilized 9 g /L of reducing sugars from *C. vulgaris* ESP6 biomass, and the cumulative hydrogen production finally reached 1476 mL/L [15]. In a batch experiment, *C. butyricum*, as the dominant species, efficiently utilized 4.9 g/L of reducing sugars to produce hydrogen of 32.0 mL/g-TVS. Furthermore, aerobic methanotrophs such as *Methylomicrobium alcaliphilum* 20Z and *Methylomonas methanica* are able to provide sugar and organic acids [20,21]; therefore, they also have the potential to serve as substrate for hydrogen production.

In this work, a co-culture of *Chlorella sacchrarophila* FACHB 4 and *Methylomonas* sp. HYX-M1 was established, and the capacity of methane oxidation was assessed at the same time. Subsequently, the co-culture biomass was digested with 3% H_2SO_4 and was analyzed by the HPLC system and excitation–emission matrix spectrometry. Furthermore, two *Clostridium* sp. were induced to produce hydrogen from co-culture biomass, without any additional organic carbon sources, under various conditions. The influence of the carbon source composition was evaluated in order to select the best production profile according to the characteristics of the microalgal biomass. Our experimental outcomes aim to bridge this gap by presenting a novel approach to valorize the combined biomass output from methanotrophic bacteria and microalgae, thereby charting a path towards sustainable resource utilization and circular bioeconomy.

2. Materials and Methods

2.1. Precultures of the Microorganism

The microalgae used in this work was *Chlorella sacchrarophila* FACHB 4 purchased from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). *C. sacchrarophila* was grown in BG-11 medium [22] and was cultivated under continuous illumination at 6000 lux and at the temperature of 30 °C.

The methanotroph strain *Methylomonas* sp. HYX-M1 was isolated from the Pearl River Estuary (Guangzhou, China). *Methylomonas* sp. HYX-M1 was grown in modified NMS medium under aerobic conditions with approximately 25% methane and was incubated in darkness at a temperature of 30 °C [23].

Clostridium pasteurianum DSM525 purchased from the DSMZ and *Clostridium* sp. BZ-1 isolated from The Pearl River Estuary were cultured in modified mineral salt glucose (MSG) medium [24]. The two microorganisms were both incubated at the temperature of 37 °C in the dark. The pH value was adjusted to 6.5 ± 0.1.

2.2. Co-Culture Experiments of Microalgae and Methanotroph

The culture solution containing *C. sacchrarophila*. FACHB 4 and *Methylomonas* sp. HYX-M1 was concentrated by centrifugation (8000 rpm, 5 min) and resuspended in 50 mL of NMS, respectively. In the following co-culture experiments, cells were grown in 125 mL

serum bottles of 25 mL of NMS sealed with a septum and aluminum cap. Cells were inoculated at a 4:1 (*Methylomonas* sp. HYX-M1: *C. sacchrarophila*. FACHB 4) ratio based on volume. In each vial, the initial OD_{680} of the co-cultures were 0.1. 25 mL air was extracted, and the 25 mL of methane was added as the only carbon substrate. All experiments were incubated in a light incubator at 30 °C under a light intensity of 6000 lux, 8000, and 12,000 lux (144 μmol/s/m^2). Single culture of *C. sacchrarophila*. FACHB 4 and *Methylomonas* sp. HYX-M1 were set as control. Three parallel controls were set for each group. An amount of 200 μL of gas sample was taken from the headspace by a sterilized syringe for gas detection. CH_4 and CO_2 were analyzed by gas chromatography (GC; Agilent 7820A, Santa Clara, CA, USA) equipped with a flame ionization detector and a thermal conductivity detector, as previously described [23].

2.3. Hydrolysis of the Biomass of Co-Cultures

The co-cultures were centrifuged at 8000 rpm for 5 min and subsequently resuspended with 10 mL distilled water. The harvested microalgal biomass was first treated with 3% H_2SO_4 under autoclave condition (120 °C for 30 min). After acidification, the solution was centrifuged at 8000 rpm for 5 min and filtrated through a 0.22 filter membrane. The supernatants under different light intensities were stored in −20 °C for the following experiments.

2.4. Fermentation Experiments

Experiment 1. To identify the hydrogen production of *Clostridium* sp. from co-culture biomass, 10 mL of acid solution was added into 10 mL of prepared MSG medium without glucose. The pH value of mixed culture medium was adjusted to 7 and then transferred into 25 mL serum bottles with a working capacity of 5 mL. These bottles were sealed with butyl rubber plugs and aluminum caps. By using equipment furnished with vacuum pumps and nitrogen cylinders, the serum bottles were deoxygenated by evacuating and flushing them with high pure N_2 to create an anoxic environment over five cycles. Then, 5% *Clostridium pasteurianum* DSM 525 (0.25 mL) and 5% *Clostridium* sp. BZ-1 (0.25 mL) were injected into the sterilized medium, respectively. The MSG medium, without a carbon source and with 5 mM glucose, 5 mM xylose, or 5 mM lactic acid, was set as control. All batch tests were kept stable at 37 °C in the dark. Hydrogen was analyzed by a gas chromatograph (GC, Agilent 7820A, USA) equipped with a thermal conductivity detector.

Experiment 2. To determine the hydrogen production of *Clostridium* sp. with co-culture biomass under various light intensities, 10 mL of acid solution cultured under various light intensities was added into 10 mL of prepared MSG medium without glucose. The pH value of mixed culture medium was adjusted to 7 and then transferred into 25 mL serum bottles with a working capacity of 5 mL. The headspace of serum bottles was replaced with nitrogen gas. Then, 5% *Clostridium* sp. (0.25 mL) was injected into the sterilized medium. All batch tests were kept stable at 37 °C in the dark.

2.5. Analysis of Soluble Metabolites

Organic acids and solubilized sugars were quantified in the cell-free supernatants by HPLC (LaChrom, Merck, Darmstadt, Germany) equipped with a guard, an analytical column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA, USA), and a refraction index (RI) detector (LaChrom L-7490, Merck, Germany). The temperature of the column and the RI detector were maintained constant at 50 °C and 45 °C, respectively. The samples were eluted with diluted H_2SO_4 at a flow rate of 0.6 mL/min. The solutions of the analyzed organic acids and sugars served as external standards.

2.6. Three-Dimensional Fluorescence Spectrum

All the samples were characterized by using three-dimensional fluorescence spectrum (Hitachi, FP6500, Tokyo, Japan). Three-dimensional excitation–emission matrix spectra were determined with scanning emission spectra from 250 nm to 550 nm at 2 nm increments

by varying the excitation wavelength from 220 nm to 500 nm at 5 nm increments, with a speed of 1200 nm/min. The blank spectrum was recorded with deionized water.

2.7. Statistical Analysis

All the batch cultures were conducted in triplicate, and the data were the mean ± standard deviation. Statistical analyses were conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) or Origin 2018. Significant differences were determined by one-way ANOVA, and a p value < 0.05 was considered statistically significant. NS presented no significant differences ($p > 0.05$).

3. Results and Discussion

3.1. Methane Consumption of Co-Cultures

The methane consumption and growth of microalgae, methanotrophs, and co-cultures were investigated, respectively. Under pure culture conditions, *C. sacchrarophila.* FACHB 4 utilized a small amount of CO_2 to grow and failed to consume methane, whereas the growth of *Methylomonas* sp. HYX-M1 was determined by the content of headspace oxygen, which could only consume 59.32% of the total methane (Figure 1a,b). In co-cultures, microalgae grew with carbon dioxide produced by methanotrophs, while microalgae produce oxygen through photosynthesis to supply the methanotroph in return [8,10]. Thus, the co-culture system was able to utilize more methane for biomass production compared to methanotroph culture alone. The highest methane utilization of the co-culture system was 1.00 mmol, which was twice as much as that of HYX-M1 cultured alone; meanwhile, the maximum OD_{680} value of the culture solution was 2.5, which was five times as much as that of HYX-M1 cultured alone (Figure 1). Similar to our study, Wang found that methane assimilation of the combined system (*Chlorella vulgaris* and aerobic methanotrophs) was 12.50% higher than that of aerobic methanotrophs cultured solely and the dry biomass was as high as 250 mg/L [25]. The highest methane consumption rate of alkaliphilic methanotrophs and *Scenedesmus obtusiusculus* co-culture in batch assays ranged from 2.5 to 19.8 mmol $CH_4/g_{biomass}/d$ [11]. The methane oxidation rate in a co-culture of methane oxidizing communities and microalgae was 10.68 mmol/L/d w, and the average methane oxidation rate of *Scenedesmus* sp. and *Methylocystis parvus* co-culture was 20.56–36.8 mmol/L/d [8,9]. The methane assimilation rate of *Chlorella sorokiniana–Methylococcus capsulatus* co-culture was approximately 8 mmol/L/d [6]. In our study, the co-culture's average methane oxidation rate was 10 mmol/L/d (9.52 mmol $CH_4/g_{biomass}/d$), suggesting that the methane oxidation rate of constructive co-culture was at a moderate level.

The pH value has always been a key factor affecting the growth of microorganisms; meanwhile, the growth of microorganisms will also cause a change in a system's pH value. By monitoring the change in the pH value of the system, the state of microbial growth can be revealed. In general, the growth of methanotrophs brings about a decrease in the pH value of the medium (Figure 1d), whereas the growth of microalgae could lead to an increase in pH value. This allowed the co-culture system to maintain a stable pH value between 7.0 and 8.0 for the first 64 h (Figure 1d). After 64 h, with the depletion of methane in the culture system, the growth of methanotrophic bacteria stopped, and the continued increase of the medium indicated that microalgae could still continue to grow at this time. However, the OD_{680} value of the system did not continue to increase after 60 h, indicating that a part of methanotroph suffered death, thus offsetting the increase in OD_{680} caused by the growth of microalgae.

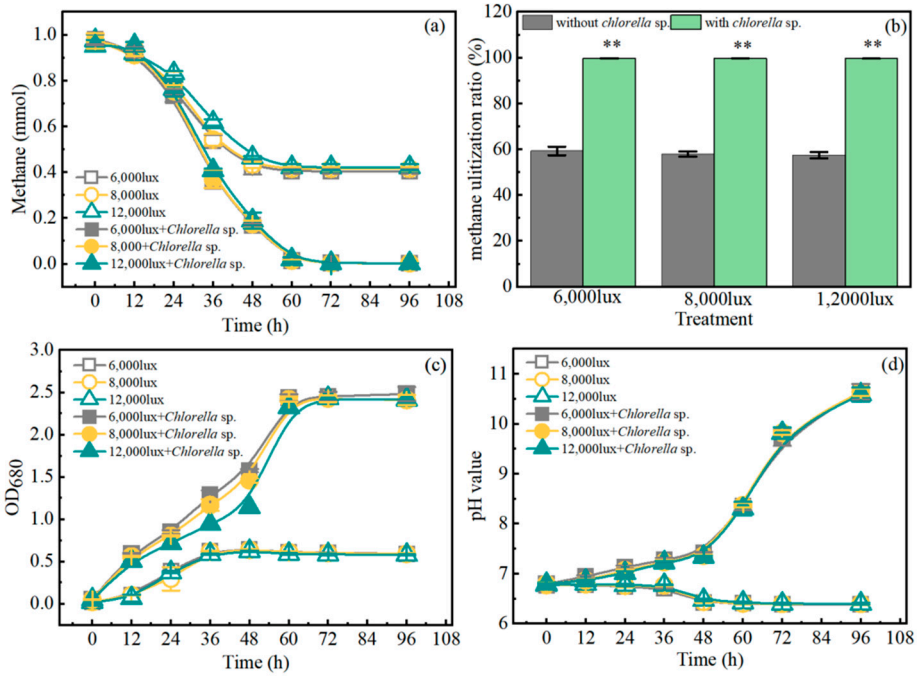

Figure 1. The dynamics of methane consumption (**a**), methane oxidation rate (**b**), and OD_{680} (**c**) and pH value (**d**) for *Methylomonas* sp. HYX-M1when cultured alone or co-cultivated with *C. sacchrarophila*. FACHB 4 under various light intensities. ** $p < 0.01$.

3.2. Metabolite Production in Co-Cultures

In this work, the co-culture biomass of microalgae and methanotroph was pretreated with 3% H_2SO_4, and then most of the glucose was released from the raw biomass into the pretreated hydrolysates. The maximum glucose production of 45.05 µmol was obtained after 4 days of batch cultivation at 12,000 lux light intensity. As the light intensity decreased from 12,000 lux to 6000 lux, the glucose yield of the co-culture hydrolysate gradually decreased, and the xylose yield gradually increased (Figure 2). The biomass obtained from 12,000 lux cultivation reached a maximum reducing sugar ratio of 13.68% (Table 1). Similarly, carbohydrate productivity from *Nannochloropsis gaditana* increased with light intensity from 6000 lux to 12,000 lux, and the carbohydrate content of *Nannochloropsis gaditana* was 21.3 ± 0.9% under 12,000 lux [26]. Sriram and Seenivasan reported that maximum carbohydrate content (25.4%) was obtained in *Desmodesmus* sp. VIT after 15 days of batch cultivation at 16,000 lux light intensity [27]. The results of the present study concluded that 12,000 lux light intensity is the optimum condition for maximum carbohydrate accumulation in co-culture. Lactic acid also accumulated in all experimental systems, and the accumulation of lactic acid gradually decreased with the increase in light intensity. Under 6000 lux light conditions, the lactic acid production of co-culture biomass was as high as 26.45 µmol (Figure 2). Some microalgae isolated from seawater, such as *Nannochlorum* sp. 26A4, had a starch content of 40% (dry weight) and a conversion rate for converting consumed starch into lactic acid of 70% [28]. Furthermore, some of the methanotrophs, such as *Methylomonas* sp. DH-1 and *Methylomicrobium alcaliphilum* 20z, were able to produce lactic acid from methane [29,30]. A dark environment is more conducive to the lactic acid fermentation of algae and the growth of methane-oxidizing bacteria, which may account for the higher lactic acid content observed under low light intensities.

In order to explore other metabolites during the co-culture of microalgae and methanotroph, the 3DEEM fluorescence spectrum of the pretreatment sample was analyzed. Amino acids such as arginine, serine and tyrosine were demonstrated to be produced by microalgae biomass after microwave pretreatment with diluted acid and aspartic acid [16]. However, no peak in the Type I and II region indicative of a protein-like peak was observed. One peak at Ex/Em of 375 nm/475 nm was observed in the 3DEEM spectra in the Type IV region.

These were humic-like peaks (Figure 2). The dehydration of polysaccharide fractions in the presence of acid catalysts is a chemical process that produces secondary humic matter. Consistent with our study, the humic acid mixture was extracted from microalgae biomass rich in polysaccharides using a standard alkali treatment [31].

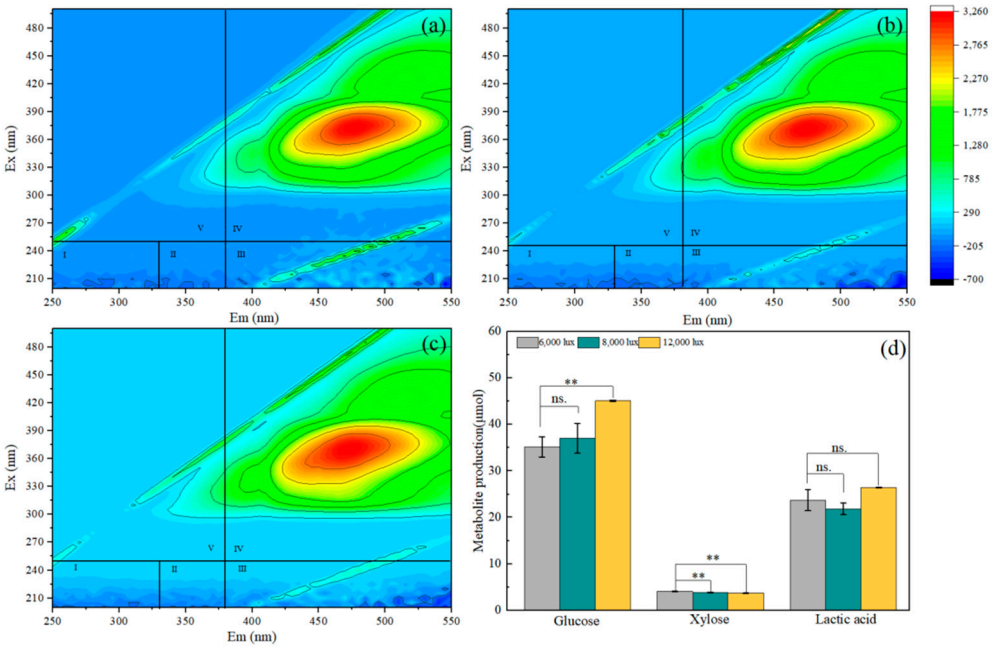

Figure 2. The three-dimensional fluorescence spectrum of co-culture biomass under different light conditions with acid treatment; (**a**) 6000 lux, (**b**) 8000 lux, and (**c**) 12,000 lux. (**d**) Metabolite production of co-culture biomass. ** $p < 0.01$; n.s. indicates that these differences are not significant. Region I and II: aromatic protein; region III: fulvic acid-like; region IV: soluble microbial by-product-like; region V: humic acid-like.

Table 1. The content of carbohydrates under various light intensities. All values were compared to the biomass cultured in 6000 lux. * $p < 0.05$, ** $p < 0.01$; n.s. indicates that these differences are not significant.

Light Intensity (lux)	Glucose (g/L)	Xylose (g/L)	Biomass (g/L)	Reducing Sugar (%)
6000	1.26 ± 0.08	0.061 ± 0.001	12.60 ± 0.42	10.55 ± 0.42
8000	(1.22 ± 0.11) **	(0.058 ± 0.001) n.s.	(12.23 ± 0.30) n.s.	(11.36 ± 0.69) n.s.
12,000	(1.62 ± 0.01) **	(0.055 ± 0.001) **	(12.26 ± 0.71) n.s.	(13.73 ± 0.84) *

3.3. Biohydrogen of Clostridium sp. from Co-Culture Biomass Hydrolysate

The pretreated biomass under 8000 lux light intensity was used as feedstock to produce hydrogen by *Clostridium* sp. during dark fermentation. Glucose, xylose, and lactic acid are the primary components of biomass [32,33], and thus, the ability of *Clostridium* sp. to utilize these substances was also tested. Strain DSM 525 failed to produce hydrogen from xylose, while strain DSM525 produced a low amount of hydrogen (0.56 μmol) from lactic acid (Figure 3). Strain DSM525 successfully generated hydrogen gas by utilizing pretreated biomass and glucose, and its maximum hydrogen production was 10.94 μmol and 27.09 μmol, respectively (Figure 3). The ability of BZ-1 to utilize glucose, xylose, lactic acid, and pretreated biomass for hydrogen production was similar to that of DSM525. Amounts of 12.26 μmol and 35.07 μmol of hydrogen were obtained by BZ-1 from pretreated biomass and glucose (Figure 3). The biomass utilization of two *Clostridium* sp. were 10.84 μmol/mL and 14.03 μmol/mL. These results suggested that selected *Clostridium* sp. were able to produce hydrogen from biomass hydrolysate, with BZ-1 exhibiting superior

hydrogen production capability. The pH value decreased to 5.5 and 6.5, respectively, in the treatment of glucose and pretreated biomass (Figure 3). The decreased pH value was attributed to the increasing accumulation of organic acids and the dissolution of CO_2 [24,34]. The *Clostridium* sp. adapted to a pH value range from 5.0 to 7.0, and, therefore, a decrease in pH value within the system was not likely to inhibit hydrogen production by *Clostridium*. Acid hydrolysis is a simple and crude hydrolysis method that may produce many toxic substances [35]. *Clostridium* sp. can directly grow and produce hydrogen by using the acid hydrolysis solution, indicating that *Clostridium* sp. have the ability to adapt to sufficient environmental resistance. This result has positive significance for industrial hydrogen production with biomass, greatly contributing to simplifying the steps and reducing costs.

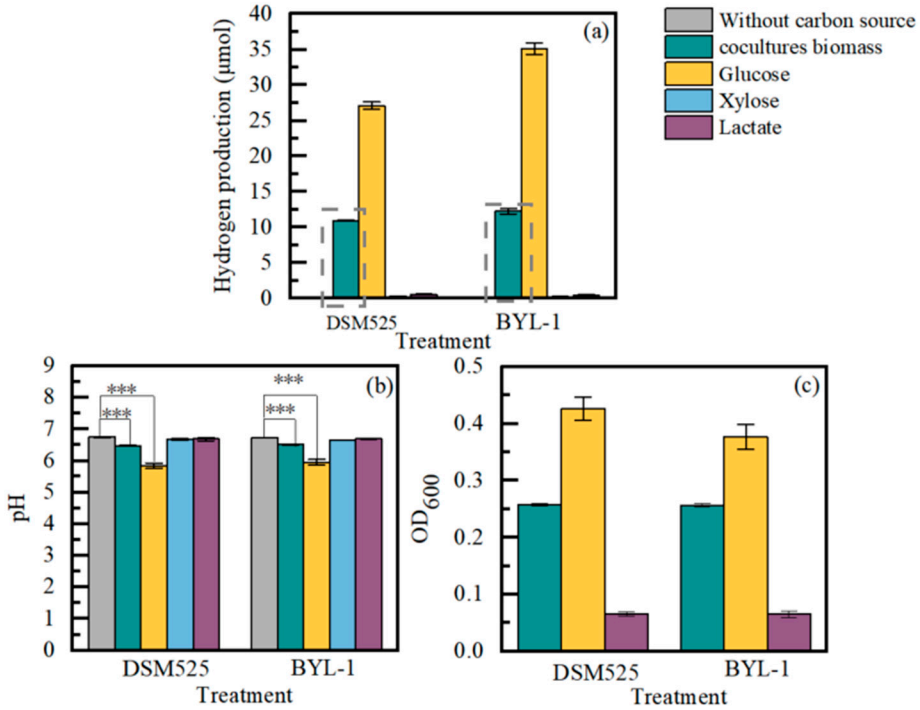

Figure 3. The dynamics of hydrogen production (**a**), pH value (**b**), and OD_{680} (**c**) of *Clostridium pasteurianum* DSM525 and *Clostridium* sp. BZ-1 with various carbon resources. The gray box represents the amount of hydrogen obtained from biomass utilization. *** $p < 0.001$.

3.4. Hydrogen Production from Co-Cultured Biomass under Different Light Intensities by Strain BZ-1

The measurement of the content of reducing sugar in the acid solution demonstrated that co-cultured biomass under 12,000 lux had the highest content of reducing sugar, and theoretically, the highest hydrogen production could be achieved by using pretreated biomass under this light intensity. However, microalgae biomass contains certain other components which were able to be utilized by *Clostridium* sp. for hydrogen production. Therefore, hydrogen production experiments with biomass acid hydrolysates under different light conditions were carried out to further verify the optimal acid hydrolysates. The cumulative hydrogen production and maximum hydrogen yield was enhanced when the biomass cultured light intensity increased. Both the highest cumulative hydrogen production (14.00 μmol) and biomass utilization (5.6 μmol/mL) occurred at the biomass cultured under 12,000 lux (Figure 4a). Similarly, *C. vulgaris* ESP6 obtained the highest hydrogen production of 1424 mL/L when the reducing sugar in biomass increased from 3 g/L to 9 g/L [15]. Thus, the hydrogen production from biomass under higher light intensity seemed to have a positive consistency with the reducing sugars in biomass. However, the lower light intensity in methane oxidation co-culture systems resulted in higher hydrogen production, and the maximum methane yield of 0.71 was detected from

co-culture biomass obtained at 6000 lux. Some components in biomass would be degraded into 5-hydroxymethylfurfural, formic acid, and levulinic acid through acid digestion [36], which are known as potential inhibitors in the following hydrogen production, leading to lower methane yield. The conversion rate of methane to hydrogen was relatively low: only 0.47%. The loss in biomass production from methane, biomass hydrolysis, and hydrogen production from hydrolysis resulted in low methane-to-hydrogen conversion. To further improve methane-to-hydrogen conversion, it is necessary to optimize the conditions for each step.

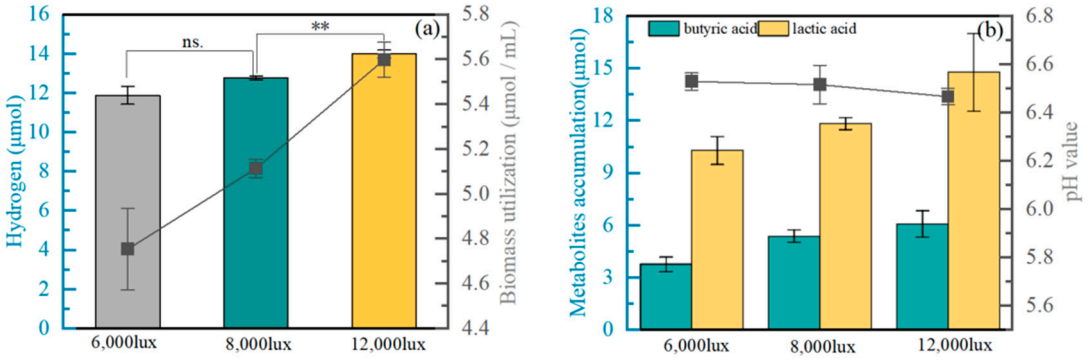

Figure 4. The hydrogen production and biomass utilization (**a**), metabolites accumulation and pH value (**b**), of *Clostridium* sp. BZ-1 with pretreated co-culture biomass incubated under different light intensities. Light intensity on the horizontal axis represents the cultivation conditions of the co-culture biomass. ** $p < 0.01$; n.s. indicates that these differences are not significant.

In the process of producing hydrogen, *Clostridium* sp. also produces organic acids, such as acetic acid, butyric acid, and lactic acid, which significantly decrease the pH value of the fermentation liquid phase, resulting in serious inhibition of hydrogen fermentation. Thus, pH value and various organic acids were detected in the experiment. The accumulation of butyric acid was enhanced when the BZ-1 strain with pretreated co-culture biomass was cultured in 12,000 lux, and the maximum production was 6.09 μmol (Figure 4b). The metabolic pathways for the production of butyric acid from glucose involved the breakdown of hexose to pyruvate and its conversion into acetyl-CoA via the Embden–Meyerhof–Parnas pathway [37,38]. Ultimately, butyric acid is produced from acetyl-CoA-derived acetoacetyl CoA via phosphotrans butyrylase and butyrate kinase. Several anaerobic *Clostridium* species, including *Clostridium tyrobutyricum*, *Clostridium* sp. S1, and *C. thermobutyricum*, are known to produce butyric acid as the main metabolite from glucose [38–40]. Moreover, accumulated lactic acid reached levels of 10.31, 11.84, and 14.80 μmol, respectively, with various pretreated co-culture biomasses. Compared to the initial system, the final lactic acid obtained in the system was slightly increased (Table 2). Therefore, the initial lactic acid in the system seemed to not be utilized as a carbon source but, rather, as a product of carbohydrate metabolism. Glucose is oxidized to pyruvate and then converted to lactate by lactate dehydrogenase. Similar to our study, researchers previously had produced approximately 8 g/L lactic acid from a PY medium with glycerol under different pH value [41]. The pH value decreased faster with increased accumulation of organic acid. Cultured with various pretreated co-culture biomasses, the pH values reached 6.52, 6.51, and 6.47, respectively (Figure 4b). Although the pH value decreased, the system remained in the range suitable for the growth of *Clostridium* sp., thereby having a limited inhibition on the hydrogen production of the BZ-1 strain.

The influence of the biomass cultured under different light intensities on the metabolism of strain BZ-1 was comprehensively illustrated based on the carbon in different cultures. Most of the carbon in the culture was retained in the biomass of the BZ-1 strain (17.07–21.03%) (Figure 5). The highest carbon (18.15%) fluxes flowing to butyrate were observed when the BZ-1 strain grew with the co-culture biomass obtained at 12,000 lux. Meanwhile, the higher light intensity in methane oxidation co-culture systems resulted

in higher carbon flows to lactate, with maximum values of 18.15%. These results indicate co-culture biomass obtained from different light intensities leads to various carbon fluxes of strain BZ-1. Overall, this study provides a new method for obtaining hydrogen from biomass with methane, but the optimization of culture conditions remains to be further explored.

Table 2. The consumption or production of glucose by strain BZ-1with pretreated biomass cultured under various light intensities.

	Light Intensity (lux)	Glucose (μmol)	Lactic Acid (μmol)	Butyric Acid (μmol)	Hydrogen Yield (μmol/μmol)
Before culture	6000	17.56	5.92	0	/
	8000	18.51	5.46	0	/
	12,000	22.52	6.61	0	/
After culture	6000	0	10.31	3.78	0.71
	8000	0	11.84	5.39	0.70
	12,000	0	14.80	6.09	0.62

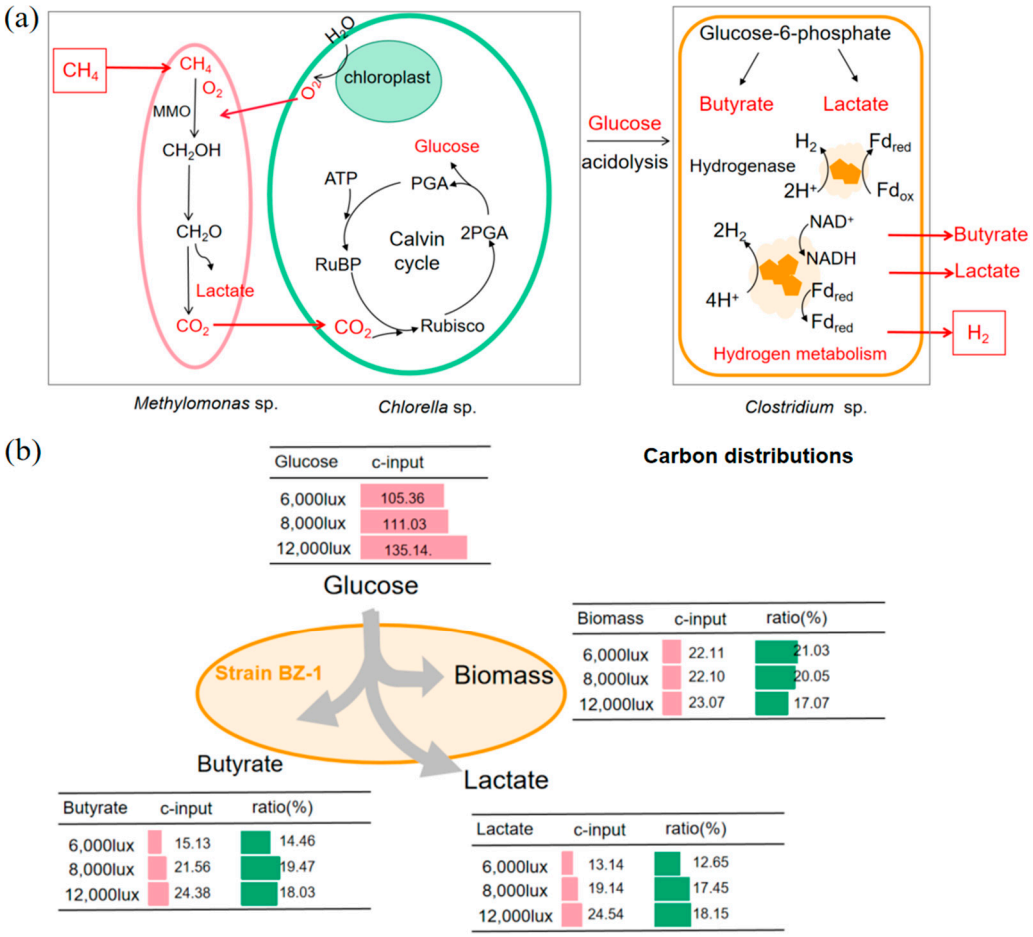

Figure 5. Mechanism of synergistic metabolism of aerobic methanotroph, microalgae, and *Clostridium* sp. to produce methane removal and hydrogen production (**a**). Aerobic methanotroph first utilizes methane to produce carbon dioxide as a substrate for microalgae growth, while microalgae provide oxygen for further methane oxidation. *Clostridium* sp. use the reducing sugar in co-culture biomass to accumulate hydrogen and organic acid. Average carbon (**b**) of the products from glucose fermentation with pretreated co-culture biomass incubated under different light intensities.

4. Conclusions

The co-culture of *Chlorella sacchrarophila* FACHB 4 and *Methylomonas* sp. HYX-M1 produced biomass from 1 mmol of methane in 96 h. Furthermore, up to 45.05 µmol of glucose, 4.07 µmol of xylose and 26.5 µmol of lactic acid were obtained from co-cultured biomass after acid digestion. *Clostridium pasteurianum* DSM525 and *Clostridium* sp. BZ-1 produce hydrogen from soluble sugars in pretreated co-culture biomass. The hydrogen production of strain BZ-1 increased to 14.00 µmol with 8.19 µmol lactate and 6.09 µmol butyrate from the co-culture biomass obtained at 12,000 lux. These findings introduce an innovative pathway for valorizing the co-produced biomass from methanotrophic microorganisms and microalgae, thereby offering a dual strategy that concurrently promotes environmental remediation and bioenergy generation.

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