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Biohydrogen production from glycerol by novel *Clostridium* sp. SH25 and its application to biohydrogen car operation

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Abstract−Biohydrogen is a clean and efficient source of energy produced easily by anaerobic systems. Therefore, the discovery of novel and efficient production methods and utilization of inexpensive starting material are crucial for economical biohydrogen production. In this study, novel hydrogen producing bacterial strain *Clostridium* sp. SH25 was screened from the anaerobic sludge obtained from a water treatment plant, which showed a higher hydrogen-producing activity on glycerol than other strains. The effective hydrogen production was evaluated under varying anaerobic culture conditions, and the optimum temperature, initial pH, additional NaCl concentration, and inoculum size were 37 °C, 6.0, 0%, and 10% (v/v), respectively. The cumulative hydrogen production volume from crude glycerol was 24.30±1.07 ml after 36 h. To test the practical application of biohydrogen, a 20 ml culture of *Clostridium* sp. SH25 was incubated for 12 h and directly applied to a small hydrogen car unit operated for 19.05±0.33 s with 8.37±0.21 m displacement. Overall, identification of the efficient *Clostridium* sp. SH25 strain resulted in the production of a large amount of biohydrogen, which further supported the operation of a small hydrogen car. This implied a possible application of biosystems in biohydrogen production.

Keywords: *Clostridium* sp. SH25, Anaerobic Screening, Crude Glycerol, Biohydrogen Production

INTRODUCTION

Fossil fuels represent an adequate and convenient source of energy. However, they have a major drawback in terms of the acceleration of global warming [1], since greenhouse gases such as CO_2 are released upon their combustion [2]. Hence, the discovery of alternative sources of energy apart from fossil fuels is one of the most prominent challenges in the 21st century [3]. General alternative sources of energy, including solar energy, hydroelectric, hydrogen, geothermal, wind, and biofuel such as biohydrogen, bioethanol are being studied and implemented [4,5]. However, renewable energy sources such as wind power and sunlight have characteristics that require more difficult energy management and storage because their energy production is not constant due to the surrounding effects [6]. On the other hand, hydrogen is one such example of alternative energy. It is regarded as one of the most potent alternative energy sources because it is renewable, has high energy yield (141 kJ/g), and is eco-friendly with only water as the by-product of its combustion [7]. Nevertheless, the conventional sources of hydrogen are neither renewable nor carbon neutral, because most of hydrogen is currently produced from fossil fuels [8]. Technologies for generating hydrogen energy from fossil fuels, the main of which are hydrocarbon reforming and pyrolysis. These methods are the most developed and commonly used [9]. Thus, it is crucial to find new ways to benefit from hydrogen as a fuel source while circumventing the greenhouse gas cost.

Biohydrogen has been gaining attention for its potential as a sustainable alternative to the existing hydrogen production methods [10]. Biological hydrogen production has been studied widely due to the mild reaction conditions and high potential environmental benefits [11]. With the advancement in this area, the hydrogen production rate has been increased, thus simplifying the construction of reactors [12]. Biohydrogen is generally produced via three methods: photosynthesis, microbial cell electrolysis, and dark fermentation. Among these, dark fermentation has exhibited remarkable potential in the last two decades because it is economical due to being simpler and cheaper than light fermentation, steam reform, or gasification [13] This method has emerged as a crucial subject of research considering the economic and environmental aspects, as it utilizes renewable feedstocks, such as bio-waste, wastewater, and biomass for biohydrogen production [14].

Biohydrogen production is achieved via anaerobic digestion [15].

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Clostridium spp. and *Enterobacter* spp. and *Bacillus* spp. [16-18] represent some of the microorganism groups that produce hydrogen through anaerobic digestion [18]. Among these, *Clostridium* spp. appears to be the most suitable, since approximately 70% of the biogas produced during fermentation is hydrogen [18-21].

Glycerol, which has an adequate pH and buffering capacity for biofuel and biochemical production, is an inexpensive by-product of biodiesel production [22,23]. With the recent increase in the popularity of eco-friendly and sustainable alternatives [24,25], the biodiesel industry is expected to expand, and a concomitant increase in the amount of glycerol produced through biodiesel is also projected. Biodiesel is produced through a process called transesterification, wherein glycerol is a by-product [26]. However, this glycerol is crude and difficult to use in fermentation without purification [27]. The impurities in crude glycerol affect the biochemical pathways in the bacterial cells and may limit the efficiency of metabolite production [28]. However, the use of crude glycerol in fermentation media without prior purification is anticipated to have a significant advantage over the conventional use of pure glycerol as substrate, and several reports have described its use as a carbon source [28-31]. Many researchers have attempted to enhance the value of crude glycerol by transforming it into chemicals such as ethanol, 1,3-propanediol, and 2,3-butanediol [29,32-35]. In fact, biohydrogen production using crude glycerol obtained from the biodiesel manufacturing industry has already been investigated [28]. However, the amount of hydrogen produced is relatively low. Therefore, more efficient producers are still required.

The aim of this study was to assess the potential of novel *Clostridium* sp. SH25 to produce hydrogen using glycerol. This strain was isolated from the anaerobic sludge obtained from the Joongnang water treatment center, and the operational fermentation conditions were determined for biohydrogen production using crude glycerol. Furthermore, the practical application of this biohydrogen was evaluated by the operation of a hydrogen car.

MATERIALS AND METHODS

1. Chemical and Isolation Source

Pure glycerol was obtained from Junsei Chemical Co. (Tokyo, Japan) and crude glycerol (80% w/w pure) was obtained from Aekyoung Petrochemicals (Seoul, Korea). All other chemicals belonged to analytical grade (Sigma Aldrich, USA), (Merck, Germany). Sludge sample was collected from the Joongnang water treatment center (Seoul, South Korea).

2. Strain Isolation and 16S rRNA Sequencing

A modified 2x YTG media, containing 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, and 10 g/L glycerol was used in this study. In addition, modified 2x YTG agar plates were prepared for screening process by adding 20 g/L bacteriological agar (Kisanbio, South Korea) to the modified 2x YTG media. The sludge sample was diluted with sterile distilled water, and then spread onto the modified 2x YTG agar plates in an anaerobic environment. The inoculated 2x YTG agar plates were then incubated under anaerobic conditions using the anaerobic chamber (Coy Laboratory Products Inc., MI, USA) containing an atmosphere of 85% nitrogen (N_2), 10% CO_2, and 5% hydrogen. After 24 h, certain types of single colonies were isolated from the incubated culture plates. Each isolated single colony was cultured separately in the modified 2x YTG media for 24 h, and then stored at −80 °C in 20% glycerol as stock solutions for further use [36].

The isolated strains obtained from the anaerobic sludge were identified at the species level using 16S rRNA sequencing through polymerase chain reaction (PCR) amplification using the primer 27F, as described previously [37]. Partial sequences were obtained by Cosmo Genetech Co., Ltd. (Seoul, South Korea) and aligned to the nucleotide sequences available in the NCBI GenBank database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [38].

3. Selection of Strains and Fermentation Condition

All isolated strains stored as glycerol stocks were inoculated on modified 2x YTG plates and incubated in the anaerobic chamber. After 24 h of incubation, a single colony of each strain was pre-cultured in 5 mL modified 2x YTG broth. All main cultures were set up in a working volume of 20 mL 2x YTG media in a 50 mL serum bottle. N_2 gas was blown through the media for 10 min to maintain the anaerobic environment, and rubber stoppers were used to prevent the biogas from escaping [39]. Before arranging the main culture for anaerobic biohydrogen production, the serum bottles and culture media were sterilized at 121 °C for 15 min using an autoclave. The preculture (2 mL) of each strain was inoculated into 20 mL modified 2x YTG media in a serum bottle for biohydrogen production. Fermentation was carried out at 37 °C and 200 rpm in a shaking incubator.

4. Optimization of Hydrogen Production by *Clostridium* sp. SH25

To find the optimal conditions for biohydrogen production, *Clostridium* sp. SH25 was cultured under various conditions with different parameters. First, an experiment was conducted to find an optimal carbon source for biohydrogen production by *Clostridium* sp. SH25. The modified 2x YTG medium was used for the optimization of the carbon source, containing 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, and 10 g/L diverse carbon sources (glucose, fructose, sucrose, galactose, glycerol and lactose). After carbon source optimization, other tested parameters included glycerol concentration (5, 10, 25, 50, 75 and 100 g/L), temperature (20, 25, 30, 37, and 45 °C), pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), additional NaCl concentration (0, 0.5, 1.0, 1.5, 2.0 and 2.5% (w/w)), and inoculum sizes (5, 10, 15, 20, 25 and 30% (v/v)). To alter the pH of the media, 5 mol/L HCl and 5 mol/L NaOH were used [33]. After the optimized condition for biohydrogen production was determined, *Clostridium* sp. SH25 was cultivated for biohydrogen production from crude glycerol via fermentation at optimal conditions.

5. Gas Chromatography (GC) Using Thermal Conductivity Detector (TCD) Operation for Biogas Analysis

To measure biohydrogen productivity, biogas analysis was conducted using gas chromatography, as follows: the volume of the biogas released by each strain under each parameter was measured using a glass syringe (Sigma Aldrich, USA) and compared. In addition, 0.2 mL of biogas was collected from the headspace and analyzed for validating the biohydrogen content in the total biogas via gas chromatography (YL6500 GC; YoungIn Chromass, South Korea) using a thermal conductivity detector with a 3.66 m×3.18 mm× 2 mm Porapak™ N packed column (Agilent Technologies, Santa

Clara, CA, USA) and N_2 as a carrier gas. The temperature of the inlet, oven, and detector of the GC system was set at 150 °C, 80 °C, and 150 °C, respectively. The analysis time was 2 min, and the hydrogen peak was identified at about 0.8 min. Hydrogen concentration in all experiments was quantified in comparison with 0.2 mL standard hydrogen gas (Scotty gases; VWR Scientific, Radnor, PA, USA).

6. High-performance Liquid Chromatography (HPLC) Operation

To determine the consumption of glycerol and the amount of organic acids produced, such as lactate, butyrate and acetate, 0.2 mL of the cultured medium was extracted from anaerobic serum bottle using a 5 mL syringe (Koreavaccine Co., Ltd., Gyeonggi-do, Korea). The extracted samples were vortexed for 5 s, followed by centrifugation at 13,000 rpm for 10 min [34]. The supernatant (100 µl) was aspirated and diluted with 900 µL HPLC grade distilled water (Fisher Scientific, USA). The diluted supernatant was filtered through a polyvinylidene fluoride membrane filter (CHROMDISC PVDF syringe filter, 13 mm, Korea) with a pore size of 0.45 µm before analysis [40]. Residual glycerol concentration and the amount of organic acids produced was assessed through high-performance liquid chromatography (HPLC; Flexar HPLC system; Perkin Elmer, Waltham, MA, USA) system, which included a refractive index detector (RID) and UV-Vis detector (UVD). UVD was operated at 210 nm. The 300 mm×7.8 mm Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA, USA) was used in this study. The mobile phase was 0.008 N sulfuric acid (H_2SO_4), administered at a flow rate of 0.6 mL/min for 40 min [36].

7. Gas Chromatography (GC) Using FID (Flame Ionization Detector) Maintained Operation for Organic Alcohol Analysis

To estimate the organic alcohol produced by *Clostridium* sp. SH25, 1 mL of the culture media was extracted using a 5 mL syringe (Koreavaccine Co., Ltd., South Korea), and centrifuged at 12,000×g for 10 min. The samples were then filtered through a polyvinylidene fluoride membrane filter (PVDF syringe filter, 13 mm, CHROMDISC, Korea) with a pore size of 0.45 µm. Thereafter, the purified samples were analyzed using gas chromatography (GC) to determine the amount of organic acid produced.

GC (YoungIn Chromass, South Korea) was performed using DB-WAX UI column (30 m×0.25 mm×0.25 µm) (Agilent Technologies, CA, USA). Analytical grade methanol (Merck, Germany) was used as the pre-wash and post-wash solvent. The filtered culture sample (5 µL) was injected into the column with a 1 : 90 split ratio. The carrier gas was helium (He), and the flow rate was fixed at 1 mL/min. The oven was maintained at 60 °C for 2 min, followed by subsequent heating to 230 °C at a rate of 15 °C/min, and then maintained at 230 °C for 3 min. Peak detection was conducted using a FID (YoungIn Chromass, South Korea) maintained at 230 °C [41].

8. Application of Biohydrogen in an Educational Model Hydrogen Car

To test the actual application of biohydrogen produced in this study, an educational model hydrogen car (Horizon Educational, Horizon Fuel Cell Europe, s.r.o., Prague, Czech Republic) was used. Before operating the hydrogen car, oxygen collection was performed using distilled water through electrolysis for about 60 s. The hydrogen produced through water electrolysis was removed by using 50 mL syringe. Then, a 50 mL serum bottle containing the culture medium of *Clostridium* sp. SH25 was connected to the hydrogen car through a tube. The released biogas was blocked using the 50 mL syringe prepared by extracting a small amount of biogas from the cultured bottle. After this process, the operating time and distance of the hydrogen-fueled vehicle was recorded.

RESULTS AND DISCUSSION

1. Isolation and Identification of *Clostridium* sp. SH25

Sludge was considered an adequate source for the isolation of biohydrogen-producing anaerobic bacteria, owing to the enriched microbial communities and an anoxic habitat [42-45]. The sludge samples have been found to contain many anaerobic bacteria such as *Clostridium* spp., *Klebsiella* spp., *Enterobacter* spp., and *Bacillus* spp. [46,47]. However, many strains have showed low hydrogen productivity when glycerol was utilized under anaerobic conditions [48]. Among the strains isolated in the present study, *Clostridium* sp. SH25 showed the highest activity for biohydrogen production (Fig. 1(a)). When the isolated strains were compared for biohydrogen production with glycerol (at 10 g/L), this strain displayed a cumulative hydrogen volume of 23.16±0.48 mL. The identity of this strain as *Clostridium* sp. SH25 was confirmed through 16S rRNA sequencing and NCBI-BLASTn. Phylogenetic analysis revealed that *Clostridium* sp. SH25 is closely related to *Paraclostridium bifermentans* strain JCM 1386 (Fig. 1(b)).

2. Assessment of Substrate Utilization for Biohydrogen Production by *Clostridium* sp. SH25

To analyze the substrate specificity of *Clostridium* sp. SH25, six types of carbon sources, including glucose, fructose, sucrose, galactose, glycerol, and lactose were evaluated for their effects on *Clostridium* sp. SH25-mediated hydrogen production. This strain was capable of utilizing a variety of substrates to produce biohydrogen. However, the highest cumulative hydrogen volume (19.70±0.85 mL) was obtained from glycerol (10 g/L; Fig. 2). Following this, the cumulative hydrogen volume obtained from fructose and glucose was recorded as 9.00±1.17 and 9.52±0.22 mL, respectively. Moreover, the highest OD was observed in glycerol and it was 1.70±0.04. The productivity of hydrogen from glycerol was 37.32±1.14 (mL H_2/L*h). Compared to glucose (16.96±1.56 mL H_2/L*h), which is about 2.2 times higher.

Analyzing the effect of initial glycerol concentration (Fig. 3(a)) showed that hydrogen production by *Clostridium* sp. SH25 was inhibited at a high concentration of glycerol (over 50 g/L). This concentration was higher than the previously reported concentrations responsible for substrate inhibition in other *Clostridium* spp. [21,39,49]. At this concentration (50 g/L glycerol), the cumulative biohydrogen volume was 45.61±1.21 mL (Fig. 3(a)), whereas the growth was highest, recorded as 1.60±0.00 optical density at 595 nm (Fig. 3(b)).

The highest biohydrogen yield was recorded as 0.81±0.03 mol H_2/mol glycerol, which was obtained with an initial glycerol concentration of 10 g/L (Fig. 3(c)), whereas the biohydrogen yield with 50 g/L glycerol was only 0.60±0.01 mol H_2/mol glycerol. Furthermore, the biohydrogen yield showed a decrease as the substrate concentration increased from 10 g/L to 100 g/L. Since *Clostridium*

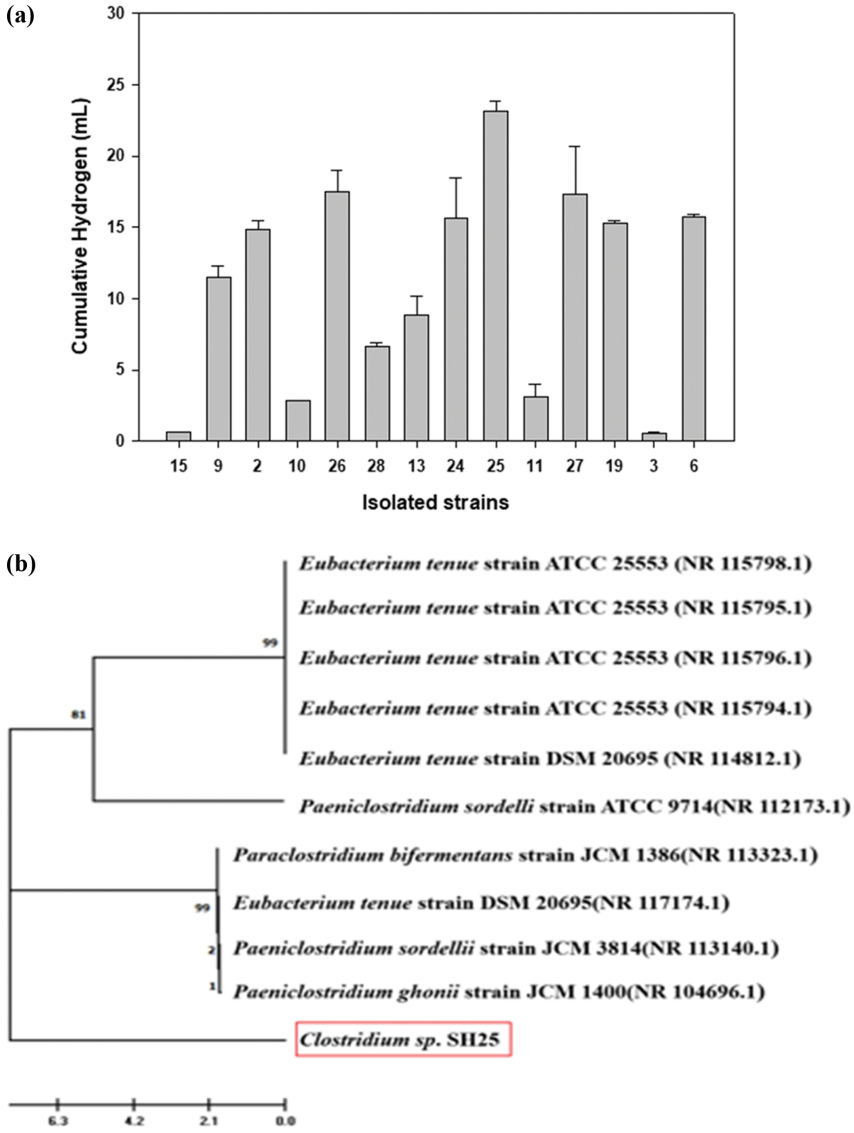

Fig. 1. Isolation and identification of *Clostridium* sp. SH25. (a) Cumulative hydrogen volume comparison among isolated strains using 10 g/L pure glycerol. (b) Phylogenetic tree depicting the relationships between *Clostridium* sp. SH25 and related species based on 16S rRNA gene.

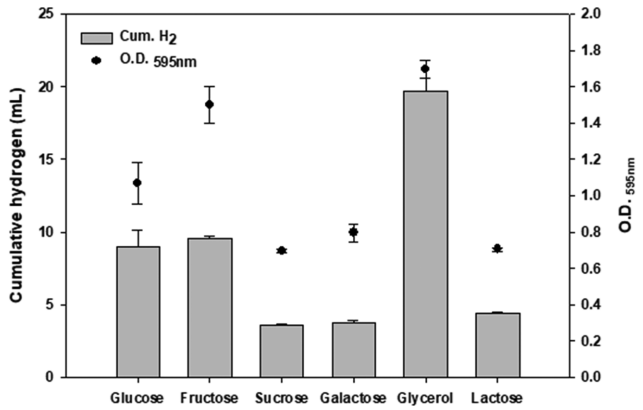

Fig. 2. Effects of different carbon sources on growth and hydrogen production of *Clostridium* sp. SH25. Effects on cumulative biohydrogen volume and Effects on optical density at 595 nm.

sp. carry out multiple catabolic pathways, several environmental conditions can induce the transition from hydrogen production to reduced fermentation, leading to end products such as ethanol [50]. In this study, as the substrate concentration was increased from 10 to 50 g/L, the hydrogen yield decreased, along with increased ethanol production. Thus, it may be speculated that the NADH accumulated due to high substrate loading results in increased ethanol production and decreased hydrogen yield (Fig. 3(d)). A similar phenomenon was observed when the influent sucrose concentration was increased from 30 to 60 g/L [51].

3. Effect of Temperature, pH, Additional NaCl Concentration, and Inoculation Size on Biohydrogen Production

To determine the optimal culture and production parameters, the effect of temperature was first examined at an initial pH of 6.5, with 50 g/L initial glycerol concentration and a 10% inoculum size. The cumulative hydrogen volume increased as the temperature

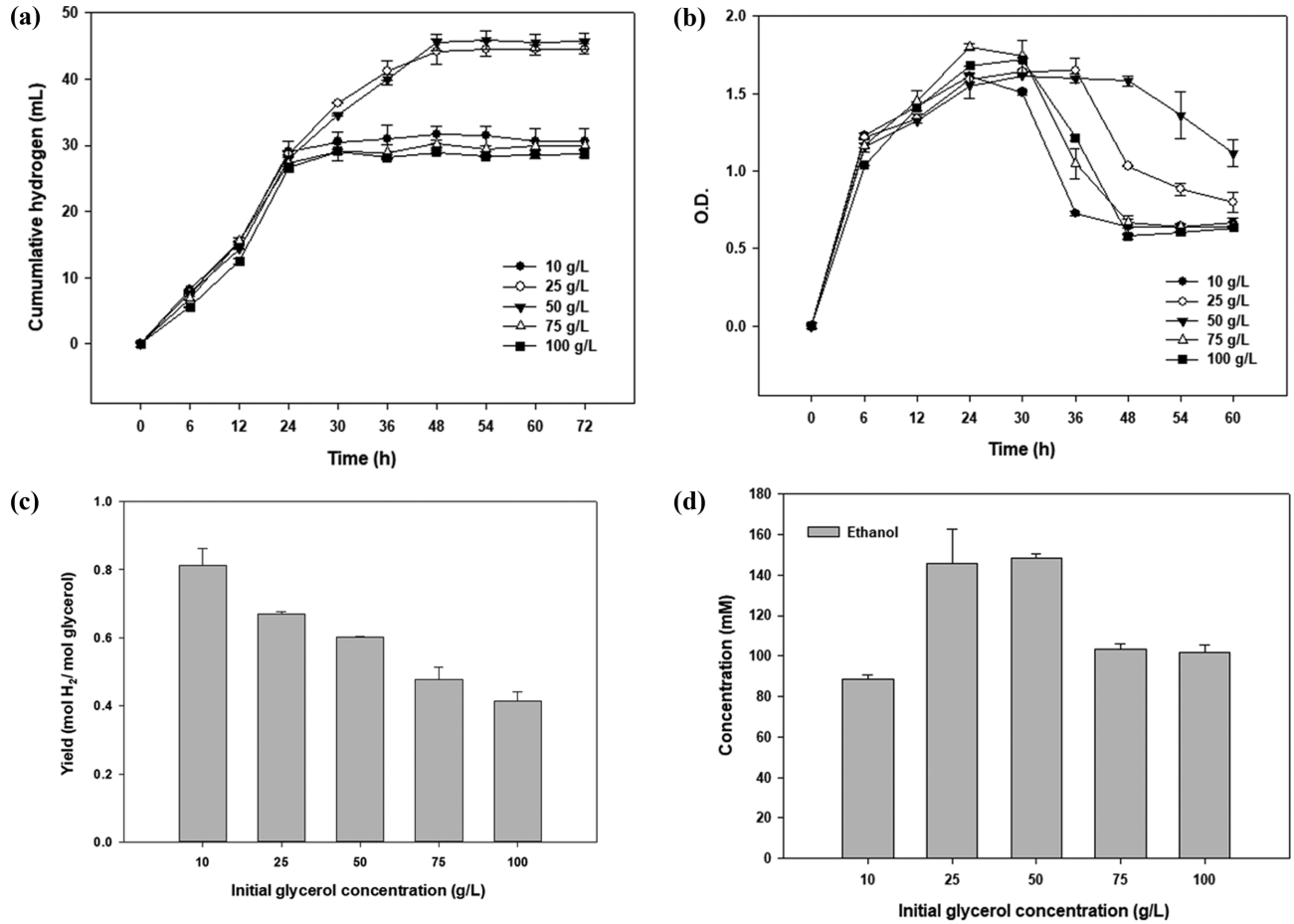

Fig. 3. Effects of initial glycerol concentration on growth and productivity of *Clostridium* sp. SH25. (a) Effects on cumulative biohydrogen volume. (b) Effects on optical density at 595 nm. (c) Effects on hydrogen yield. (d) Effects on the amount of by-products in fermentation.

was raised from 25 °C to 37 °C (Fig. 4(a)), and the highest value was recorded at 37 °C. This pattern has also been observed in other *Clostridium* spp., which display the highest hydrogen production between 35 °C and 40 °C [39,52-55].

Initial pH affects both the potential and rate of hydrogen production [56], and the hydrogen production by *Clostridium* sp. is completely inhibited in the pH range of 4 to 5 [55]. In addition, these organisms showed low hydrogen production volume at a lower initial pH [39]. Consistent with these findings, no hydrogen production was observed at initial pH 4.0; however, the cumulative hydrogen production increased concomitantly with pH and reached its maximum (31.38±0.82 mL) at pH 6.0 (Fig. 4(b)). Since the pH of crude glycerol varies in the range of 4.5-7.0 approximately, depending on the biodiesel production process [56], the *Clostridium* sp. SH25 strain is suitable for hydrogen production using crude glycerol.

Crude glycerol contains certain salts, such as 0.5 to 2.0% of NaCl or KCl [64], Therefore, the effect of NaCl concentration on biohydrogen production was evaluated. Cumulative hydrogen volume decreased as the additional NaCl concentration was increased (Fig. 4(c)); a remarkable decrease in hydrogen production was noted at 2.0% of NaCl or more.

Furthermore, the impact of inoculum size on hydrogen production by *Clostridium* sp. SH25 was evaluated (Fig. 4(d)), and the highest cumulative hydrogen volume was observed with a 10% inoculum. Since proper inoculation size is crucial for rapid initiation and a higher hydrogen production rate of a fermentative hydrogen production system [57], this finding may be applied in further experiments.

4. Biohydrogen Production by *Clostridium* sp. SH25 from Crude Glycerol

Biohydrogen production by *Clostridium* sp. SH25 was performed with pure glycerol and crude glycerol as independent carbon sources under optimized conditions, i.e., at 37 °C, with initial pH 6.0 and 10% inoculum size, and without additional NaCl. HPLC analysis revealed that crude glycerol contained 80% of pure glycerol. Thus, the glycerol content was set at 50% for both glycerol samples, and the cumulative hydrogen volume obtained with both substrates was compared. The pure glycerol substrate showed a 12% higher cumulative hydrogen volume than crude glycerol after 60 h (Fig. 5). However, the proportion of hydrogen in biogas was higher in fermentation with crude glycerol than with pure glycerol after 36 h. Consequently, *Clostridium* sp. SH25 showed a maximum biohydrogen production of 1.39±0.01 mL H_2/mL substrate from pure glycerol and that of 1.22±0.05 mL H_2/mL substrate from crude glycerol. Crude glycerol normally contains alcohol and salt contamination,

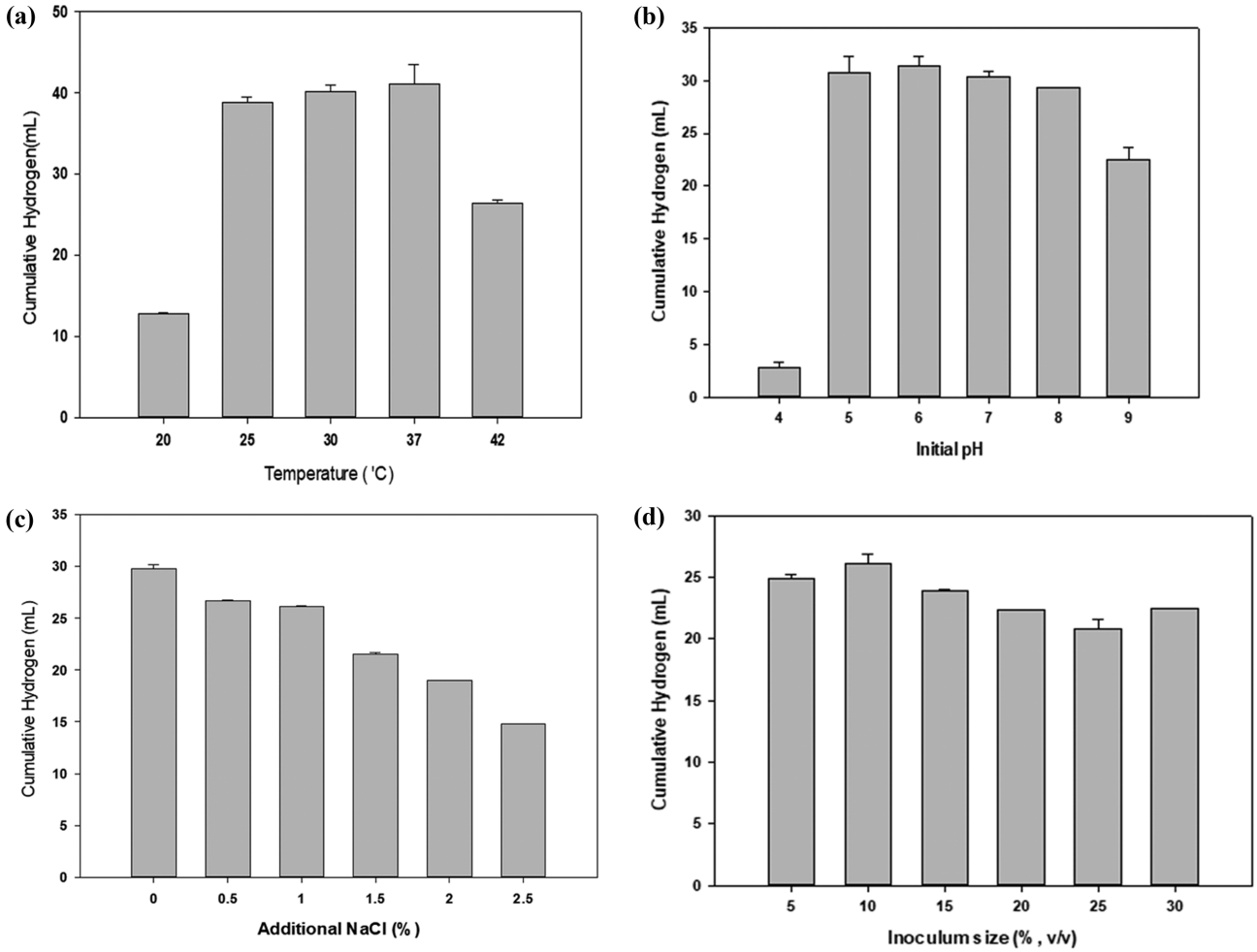

Fig. 4. Optimization of various parameters for biohydrogen production by *Clostridium* sp. SH25 from 50 g/L pure glycerol. Comparative analysis of the cumulative hydrogen volume obtained (a) at various fermentation temperatures; (b) by varying initial pH; (c) at various additional NaCl concentrations; and (d) with various inoculum sizes.

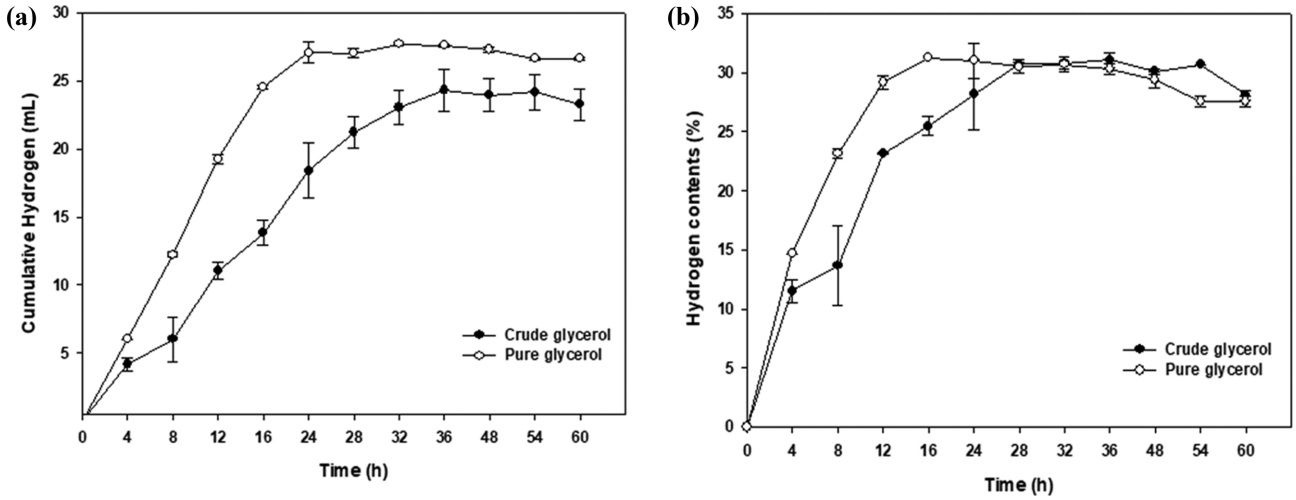

Fig. 5. Comparative analysis of biohydrogen production by *Clostridium* sp. SH25 from pure and crude glycerol under optimized condition. (a) Cumulative hydrogen volume. (b) Hydrogen content in biogas.

which results from the process of biodiesel production involving methanol and sodium methoxide [58,59]. Hence, the biohydrogen yield of *Clostridium* sp. SH25 obtained from crude glycerol was comparable to that obtained from pure glycerol, suggesting the util-

Table 1. Maximum biohydrogen production by different strains using glycerol as a carbon source

Strain	Maximum hydrogen yield	Type of fermentation	Reference
Thermotoga neapolitana DSM 4359	2.73 mol-Hydrogen/mo1-glycerol	Batch	[60]
Clostridium pasteurianum CH5	0.26 mol-Hydrogen/mol-glycerol	Batch	[64]
Clostridium pasteurianum CH7	0.23 mol-Hydrogen/mol-glycerol	Batch	[64]
Klebsiella sp. HE1	0.14 mol-Hydrogen/mol-glycerol	Batch	[64]
Clostridium acetobutylicum ATCC824	0.24 mol-Hydrogen/mol-glycerol	Batch	[65]
Clostridium butylicum DSM 2578	0.34 mol-Hydrogen/mol-glycerol	Batch	[32]
Clostridium sp. SH25	0.85 mol-Hydrogen/mol-glycerol	Batch	Present study

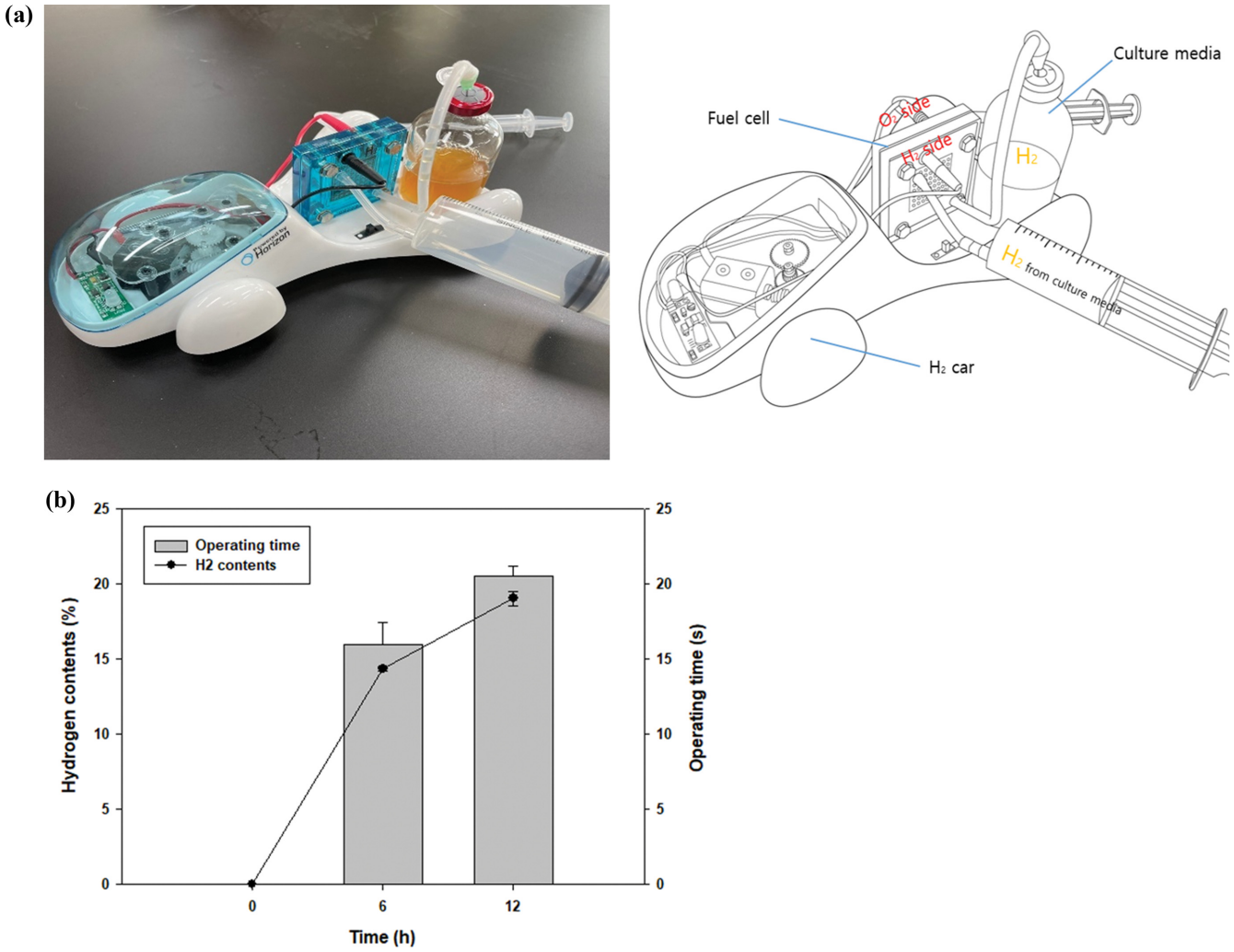

Fig. 6. Semi-application to educational model hydrogen car using biohydrogen produced (a) in this study; (b) hydrogen content (%) in biogas and operation time using biohydrogen-producing *Clostridium* sp. SH25 cultures grown for various durations.

ity of *Clostridium* sp. SH25 for hydrogen production from waste by-products. Thus, crude glycerol obtained during biodiesel production may be used in biohydrogen production even without certain recovery steps to reduce substrate costs.

The amount of hydrogen produced by *Clostridium* sp. SH25 was relatively high, when compared to other reported strains (Table 1). In contrast with *Thermotoga neapolitana*, which was able to produce 2.73 mol H_2/mol glycerol at a high temperature [60], *Clostridium* sp. SH25 required mild growth conditions for producing high amount of biohydrogen using batch fermentation.

5. Application of Cultured Bottle of *Clostridium* sp. SH25 to Model Hydrogen Car

To explore the possible applications of biohydrogen and to examine whether it could be utilized in known fuel cell systems, the biohydrogen produced in this study was applied to an educational model hydrogen car (Horizon educational, Czech Republic) (Fig.

6(a)). This car converts hydrogen into electricity using a polymer electrolyte membrane or proton exchange membrane fuel cell (PEM fuel cell) [61]. The biohydrogen produced was delivered to the anode side of the membrane electrode assemblies (MEA) and catalytically split into protons and electrons at the anode side [62]. The newly generated protons permeated through the polymer electrolyte membrane to reach the cathode side. The electrons traveled along an external load circuit to reach the cathode side of the MEA, thus creating the current output of the fuel cell crucial for the functioning of the hydrogen car [63]. In this study, a culture bottle containing *Clostridium* sp. SH25 culture was used as the hydrogen fuel tank, which supplied hydrogen gas to the vehicle. A syringe was used to administer a reverse pressure to prevent the gas from moving back (Fig. 6(a)).

Before operation of the hydrogen car, 20 mL of *Clostridium* sp. SH25 was cultured for 12 h using 50 g/L of crude glycerol, and bottles with cultures grown for 0 h, 6 h, and 12 h were prepared. Biogas was used to operate the hydrogen car and the operational time and distance was measured. The longest operation time of the car was recorded as 19.05±0.33 seconds using biogas produced from the 12 h-cultured bottle of *Clostridium* sp. SH25, and a displacement of 8.37±0.21 m was attained (Data not shown). Although the 24 h and 36 h samples were not tested, a 30% increase in operation time is anticipated based on the amount of hydrogen production. Thus, the possible application of the biohydrogen produced from *Clostridium* sp. SH25 is well demonstrated, along with the evidence that biohydrogen may also be applied to fuel cells that normally use highly pure hydrogen obtained from water electrolysis. The study also demonstrated a straightforward process for the production of biohydrogen from crude glycerol by *Clostridium* sp. SH25.

CONCLUSIONS

Clostridium sp. SH25 was isolated and identified from anaerobic sludge obtained from a wastewater treatment plant. Glycerol was determined as the best carbon source at the optimal initial concentration of 50 g/L for biohydrogen production by *Clostridium* sp. SH25, and the highest hydrogen yield was 0.81±0.03 mol H_2/mol glycerol. The optimal temperature, pH, additional NaCl concentration, and inoculum size for biohydrogen production was 37 °C, 6.0, 0%, and 10% (v/v), respectively. Under these optimized conditions, *Clostridium* sp. SH25 was able to yield 1.22±0.05 mL H_2/mL media of biohydrogen from 50 g/L crude glycerol, signifying its suitability for industrial applications. Moreover, the proportion of hydrogen in biogas obtained from crude glycerol was higher than that from pure glycerol after 36 h of fermentation. In addition, the potential application of biohydrogen derived from crude glycerol was demonstrated in a real hydrogen car. In conclusion, *Clostridium* sp. SH25 successfully produced biohydrogen using crude glycerol for application as fuel in hydrogen cars.

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