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Contributed Paper

Biohydrogen Production by Extremely Halophilic Bacteria from the Salt Pan of Samut Sakhon, Thailand

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Abstract

The optimum conditions for biohydrogen production from glucose with extremely halophilic bacteria from Samut Sakhon salt pan, Thailand were identified. The extremely halophilic bacteria's ability to produce hydrogen in nearly saturated NaCl concentration provides a potential benefit of cutting the cost of water and sterilization in dark fermentation process following alkaline pretreatment of lignocellulosic biomass. A hydrogen molar yield of 1.45 mol H_2/mol glucose at optimum conditions of 26% NaCl, 35°C, and pH 9 was obtained. The acclimatized bacteria were able to ferment various types of lignocellulosic-derived sugars. Through PCR-DGGE, the predominant hydrogen producer among the mixed culture was found to be *Halanaerobium fermentans*. These findings provided insights into the application of extremely halophilic bacteria for biohydrogen production.

Keywords: biohydrogen, extremely halophilic bacteria, *Halanaerobium fermentans*, dark fermentation

1. Introduction

As the demand for energy continues to grow, several countries worldwide have taken the measures to shift to a hydrogen economy and society, and others have started taking action according to the hydrogen fuel initiative. Currently, renewable sources only contribute to 4% of the world's total hydrogen production [1], the other 96% of hydrogen production comes primarily from fossil fuel sources [2], such as natural gas reforming and coal gasification. As the global trend moves towards renewable energy sources, hydrogen production should follow the lead. Among several methods to produce hydrogen from renewable sources by biological pathways, biohydrogen production by dark fermentation has been considered as a practically feasible option [3].

LAPORAN PENELITIAN

"Biohydrogen production by extremely halophilic bacteria from the salt pan of Samut Sakhon, Thailand"

DYAH ASRI HANDAYANI TAROEPRATJEKA S.T, M.T

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Lignocellulosic biomass, e.g., woods and grasses found in nature, is available in great quantities as biomass resource for dark fermentation, as residues from forestry, food and agricultural industries and in municipal solid wastes [4,5]. However, the structure of lignocellulosic biomass, which consists of cellulose, hemicellulose, and lignin, is naturally recalcitrant. Hemicellulose and cellulose can be biologically utilized after the application of several stages of unit processes, as follows [6]: size reduction through mechanical means, pretreatment to make cellulose more readily available for enzyme conversion, enzymatic saccharification of cellulose and hemicellulose to hydrolyze into fermentable sugars, microbial fermentation involving conversion of fermentable sugars into fuels by bacteria, and product recovery and purification. Among those processes, pretreatment has been considered as one of the most costly [7], although alkaline pretreatment with NaOH is considered as the most widely used and cost-effective for lignocellulosic biomass [6].

Extremely halophilic bacteria can reduce water use in the dark fermentation process of pretreated lignocellulosic biomass since they're capable of producing hydrogen under high conditions of NaCl. After alkaline pretreatment with NaOH, followed by neutralization with HCl to a suitable pH condition, fermentation processes to produce hydrogen can continue readily without the need for washing the pretreated lignocellulosic material with water. The anaerobic process can also help to cut the cost of electricity and devices needed for aeration. The halophilic environment is beneficial for preventing the growth of hydrogenotrophic methanogens, which have never been reported to occur at the salt concentration of 120 g/L or more [8].

Biohydrogen production by halophilic bacteria has been reported in several previous studies. Most halophiles capable of producing biohydrogen have been reported to be from the *Halanaerobium* genus. For example, *Halanaerobium hydrogeniforms* has been described to produce hydrogen up to 2.42 mmol H_2/mmol glucose [9]. Meanwhile, *H. salinarius* could produce 2 mM hydrogen from 4.17 mM glucose substrate in 14-15% NaCl at pH 7.4-7.8 [10]. Additionally, *H. chitinovorans* was reported to have the ability to produce 144. 5 μmol H_2 from 5 mL 0.5% glucose medium at 37°C and 12% NaCl [11]. Hydrogen production from glycerol by *H. saccharolyticum* subspecies *senegalensis* and *saccharolyticum* were reported to give a yield of 1.6 and 0.6 mol H_2/mol glycerol, respectively [12]. Very few studies have reported biohydrogen production in extremely halophilic condition (200-300 g/L salinity) with mixed culture.

This study aimed to determine the optimum pH, temperature, and salinity for biohydrogen production from glucose by extremely halophilic bacteria isolated from the Samut Sakhon salt pan, in preparation for biohydrogen production from lignocellulosic biomass. Additionally, the ability of the bacteria to ferment various types of carbohydrates was examined, and identification of the predominant hydrogen producer(s) among the acclimatized mixed culture of the Samut Sakhon salt pan was attempted.

2. MATERIALS AND METHODS

2.1 Inoculum and Substrate

Microorganisms collected from the soil of a commercial salt pan field in Samut Sakhon, Thailand and cultivated in a 500 mL serum bottle under 26% (w/w) NaCl (6 M or 351.35 g/L) were used as a seed inoculum. The substrate for biohydrogen production experiments was prepared as described by Taroepratjeka et al. [13] with the exclusion of $NiCl_2 \cdot 6H_2O$. The glucose concentration was adjusted at 10 g/L. All chemicals used in this study were acquired from Wako Pure Chemical Industries, Ltd. (Japan), except where otherwise stated.

Due to the opacity of the mixed culture, it was not possible to observe the bacteria's concentration through optical density method. Therefore, a different method to approximate the seed inoculum's growth phase was employed. The

substrate of the cultivation bottle was changed periodically by removing the suspension with a glass syringe and separate the biomass and the liquid through centrifugation at 12000 rpm, 25°C, for 10 minutes (Himac CF15RN; Hitachi Koki Co. Ltd., Japan). The supernatant was discarded, while the biomass pellets were returned to the cultivation bottle along with the fresh substrate. The cultivation bottle was kept at 37° C in an incubator (MIR-262; Sanyo Electric Co. Ltd; Japan). After the substrate change, biogas production in the cultivation bottle was measured and removed daily with a glass syringe. A biogas production pattern was established over time. The peak biogas production was reached after 12 days; therefore, the microbial suspension was used as an inoculum for hydrogen production experiment at day 13.

2.2 Batch Experiment for Hydrogen Production

Batch experiments were carried out in 70 and 75 mL serum bottles in duplicates for each condition. Each serum bottles were filled with 15 mL inoculum from the cultivation bottles plus 15 mL of the substrate. The bottles were capped with butyl rubber stoppers and aluminum caps, and the headspace was replaced with nitrogen gas. The experiments to determine optimum pH and temperature were performed in the presence of 26% NaCl. The initial pH was measured with a pH meter (D-51; Horiba Co. Ltd. Japan) and adjusted with NaOH (0.1, 1, or 3 M) and HCl (0.2 M).

To determine the optimum pH condition, the pH of the substrate was adjusted to 7.5, 8, 9, 9.5, or 10. The shaking incubator (BT 300; Yamato Scientific Co. Ltd., Japan) was set at 37°C with a shaking speed of 100 rpm. For analysis of the optimum temperature, the pH was adjusted to 9. The temperatures of the shaking bath incubators (BT 100 and BT 300; Yamato Scientific Co. Ltd., Japan) were set at 35°C, 37°C, 40°C, 42°C, or 45°C. For analysis of the optimum salt concentration, the substrates were prepared with 15%, 20%, or 26% NaCl. The pH was adjusted to 9, and the temperature was set to 35°C.

The volume of the produced biogas from each vial was measured and removed periodically with 5-to 50-mL glass syringes. H_2, N_2, CH_4, and CO_2 gas compositions in the vials were measured following volume measurement with gas chromatography (GC-8APT/TCD; Shimadzu Corp., Japan), using a 60/80 activated charcoal mesh (Shimadzu Corp., Japan). Argon was used as a carrier gas at the pressure of 600 kPa. The operational temperatures were 50°C for injector, 60°C for column, and 50°C for the detector, with the current set at 80 mA. The hydrogen gas volume was calculated to STP volume by multiplying the measured gas volume by the dry biogas factor for the corresponding temperature according to the method proposed by Richards *et al.*[14].

Volatile fatty acids (VFAs) were measured with gas chromatography (GC-8APF/FID; Shimadzu Corp. Japan) and a Unisole F-200 30/60 glass mesh column (Shimadzu Corp., Japan). The VFAs were measured against a standard consisted of 1000 mg/L COD each of acetic acid, propionic acid, isobutyric acid, n-butyric acid, isovaleric acid, and n-valeric acid. Nitrogen was used as a carrier gas at the pressure of 600 kPa. The operational temperatures were 250°C for injector, 140°C for column, and 140°C for detector, with the current set at 80 mA. Volatile Suspended Solids (VSS), which represented the microorganism concentration in the sample were determined according to *Standard Methods* [15].

2.3 Carbohydrate Fermentation Test

Phenol red fermentation broth was used to determine the microorganism's ability to ferment carbohydrates and consisted of 10 g/L trypticase peptone (BBL, Becton Dickinson, USA), 1 g/L beef extract (MP Biomedicals, Inc., USA), 0.018 g/L phenol red (7.2 ml of 0.25% phenol red solution), 10 g/L carbohydrate, and 351.35 g/L NaCl, made up in distilled water. The salinity of the broth was adjusted to 26% NaCl to support the acclimatized seed microorganisms. The broth without carbohydrates was prepared as a 1.25-fold

solution and autoclaved (HA-300MIV, Hirayama Manufacturing Corp., Japan). Carbohydrates were prepared separately and added to the autoclaved broth using 0.2-μm syringe filters (Minisart RC, Sartorius AG, Germany), except for starch and α-cellulose.

Each carbohydrate was tested in triplicate in 10-mL serum bottles under a nitrogen headspace. For each set of carbohydrates, one negative control with no addition of seed microorganisms was also prepared. The fermentation was observed up until three weeks of incubation, owing to the slow growth of the extremely halophilic anaerobic fermenter bacteria. Phenol red color change from red to yellow indicated positive carbohydrate fermentation. Biogas production was measured with a glass syringe from each bottle after the color change.

2.4 Theoretical Hydrogen Production and Hydrogen Molar Yield

Theoretical hydrogen production was based on these following equations for acetic acid (HAc) and butyric acid (HBu) pathways:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COO^- + 2HCO_3^- + 3H^+ + 2H_2 \quad (2)$$

Following equation (1), 4 mol hydrogen will be produced from 1 mol glucose. Hence, 498 mL of hydrogen will be produced from 1 g glucose at standard temperature and pressure (STP) conditions through the HAc pathway. For the HBu pathway in equation (2), 2 mol hydrogen will be produced from 1 mol glucose. Therefore, 249 mL H_2 will be produced from 1 g glucose through the HBu pathway at STP conditions.

The efficiency of the fermentative process is compared with the hydrogen molar yield (HMY), which is the value of cumulative hydrogen produced (mol H_2) divided by glucose provided in the substrate (mol glucose). Theoretically, the maximum HMY for glucose in anaerobic fermentation is 4, which can be reached through the HAc pathway (Equation 1).

2.5 PCR-DGGE

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) was performed according to the methods of Kongjan *et al.* [16] and Schäfer and Muyzer [17]. Genomic DNA was extracted and purified with an ISOIL for Beads Beating kit (Nippon Gene Co., Ltd., Japan). Two-step nested PCR was used to ensure species contributing less than 1% to the total population could be detected with DGGE.

The first step of the PCR to amplify the 16s rRNA was carried out using the universal bacteria primers of 27F and 1492R (Eurofins Genomics Inc., Japan). EmeraldAmp PCR Master Mix (Takara Bio Inc., Japan) was used to prepare the PCR mixture. Following PCR, the products were checked with electrophoresis (Mupid-2plus system, Mupid Co., Ltd., Japan) with 1% Agarose (L03, Takara Bio Inc., Japan), and analyzed using a ChemiDoc XRS system (Bio-Rad Laboratories, USA).

The amplicon from the first step of PCR was used as a DNA template in the second step of PCR to amplify the V3 region of the 16s rRNA with primer pairs 341F-GC and 518R. The PCR product of the second step of PCR was electrophoresed on 2% Agarose S (Nippon Gene Co., Ltd., Japan.), and analyzed.

DGGE analysis using the PCR amplicon obtained from the second step was performed using a DCode Universal Mutation Detection system (Bio-Rad Laboratories, USA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30-70% (100% denaturing solution contained 7 M urea and 40% [v/v] formamide). Electrophoresis was performed for 16 h at 70 V and 60°C in 1 × TAE buffer. SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Thermo Fisher Scientific, USA) was used to stain the polyacrylamide gel, which was then analyzed on a ChemiDoc XRS system (Bio-Rad Laboratories, USA) and documented

with Quantity One software (version 4.6.5; Bio-Rad Laboratories, USA).

Several bands that were considered important were excised from the gel and incubated with 50 μL nucleic acid-free water at 4°C overnight. The supernatants were used as a DNA template and re-amplified with 341F and 518R primer pairs. Following PCR, the amplicons were cleaned using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co, Germany) and sent to Yamaguchi University Center for Gene Research for Sanger sequencing. Alignments for phylogenetic analysis were made using MUSCLE. NCBI's MEGABLAST was used to identify the closest match for the V3 region of the 16s rRNA's gene sequence (194 bp) in the GenBank data base. A phylogenetic tree was constructed using the neighbor-joining method using the MEGAX software. The bootstrapping analysis was carried out with 1000 replicates to estimate the confidence of tree topologies.

Later on, the inoculum was grown on a GYP agar medium specified by Kobayashi *et al.* [18] to isolate and purify the species. This measure was taken to confirm the MEGABLAST result of the PCR-DGGE. PCR of 16S rRNA with 27F and 1492R primers and Sanger sequencing was then performed to colonies growing on the GYP agar. The EZBioCloud database for 16S rRNA was used as a comparison for the BLAST result.

3. RESULTS AND DISCUSSION

3.1 Optimum Conditions for Biohydrogen Production

Biohydrogen production and HMY for different temperatures and pH conditions in the presence of 26% NaCl are shown in Table 1. Only very little biohydrogen was produced at 37°C and pH 9.5 or 10. However, at pH 7.5, 8, and 9, the HMY and cumulative biohydrogen production did not differ much for conditions with the same salinity and temperature (Figure 1). In a separate experiment, HMY at pH 8.5 was also confirmed to be similar to that at pH 9. The highest HMY at 37°C and 26% NaCl was observed at pH 9 (1.20 mol H_2/mol glucose). Therefore, subsequent experiments for identification of the optimum temperature and salinity were performed at pH 9. The VFAs measurements also showed the highest acetic acid and butyric acid production at pH 9 (Figure 2), which corresponded with the HMY values. As a comparison, extreme halophiles such as *H. lacusrosei*, which can live in NaCl concentrations of 7.5–34%, show optimum growth at pH 7 [19]. On the other hand, *H. alcaliphilum* can live under conditions of pH values up to 10; however, the range for NaCl concentrations is only 2.5–25% [20].

In this experiment, the highest HMY of 1.45 mol H_2/mol glucose was produced under the conditions of 26% NaCl (351 g/L), 35°C, and pH 9 (Table 1). This value was considerably higher than the amount of biohydrogen produced by mixed culture reported by Pierra *et al.* [21] at 0.9 ± 0.02 mol H_2/mol glucose (Table 2). Figure 3 shows the cumulative biohydrogen values at different temperatures. As a comparison, the highest published HMY for halophiles was 2.42 mol H_2/mol glucose produced by *H. hydrogeniformans* in the presence of 70 g/L NaCl [9]. This value was considerably lower than that reported through dark fermentation (3.9 mol H_2/mol glucose) by *Enterobacter cloacae* [22]. The high HMY produced by *Enterobacter cloacae* was reached using a strategy in which the H_2 partial pressure was decreased from 760 mm Hg to 380 mm Hg in the head space of a batch fermentation reactor [22]. Biohydrogen synthesis pathways are prone to high partial pressures of H_2, and when end-product inhibition occurs, the pathway will shift away from biohydrogen production to lactate, ethanol, or butanol production [23].

Experiments with lower NaCl concentrations (15% and 20%) yielded steeper curves at the beginning but eventually resulted in lower cumulative hydrogen and HMY than that in the presence of 26% NaCl, (Figure 4 and Table 1). These results could be related to the pre-adaptation of the bacteria to high NaCl concentrations. Extremely halophilic bacteria manage to live in the presence

Table 1. Biohydrogen production at various pH, temperature and NaCl concentration.

NaCl	T (°C)	pH	Inoculum concentration (mg-VSS/L)	Biohydrogen production (mL)	Theoretical H_2 production		HMY (mol H_2/ mol glucose)
					HAc pathway	HBu pathway	
26%	37	7.5	334	17.02	23%	46%	0.91
26%	37	8	334	18.37	25%	49%	0.98
26%	37	9	334	18.55	25%	50%	0.99
26%	37	9.5	334	0.07	0%	0%	0.00
26%	37	10	334	0.01	0%	0%	0.00
26%	35	9	452	24.59	33%	66%	1.32
26%	40	9	452	21.55	29%	58%	1.16
26%	42	9	452	19.79	26%	53%	1.06
26%	45	9	452	20.60	28%	55%	1.10
26%	35	9	432	26.97	36%	72%	1.45
20%	35	9	432	23.57	32%	63%	1.26
15%	35	9	432	21.88	29%	59%	1.17

Fermentation period: 5-9 days. Volume was measured as STP.

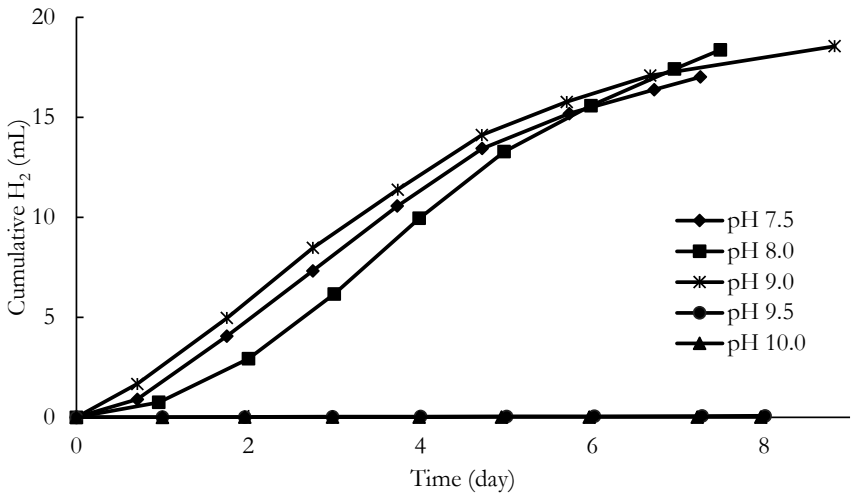

Figure 1. Cumulative biohydrogen production at different pH conditions (Temperature 37°C, 26% NaCl. Volume was measured as STP).

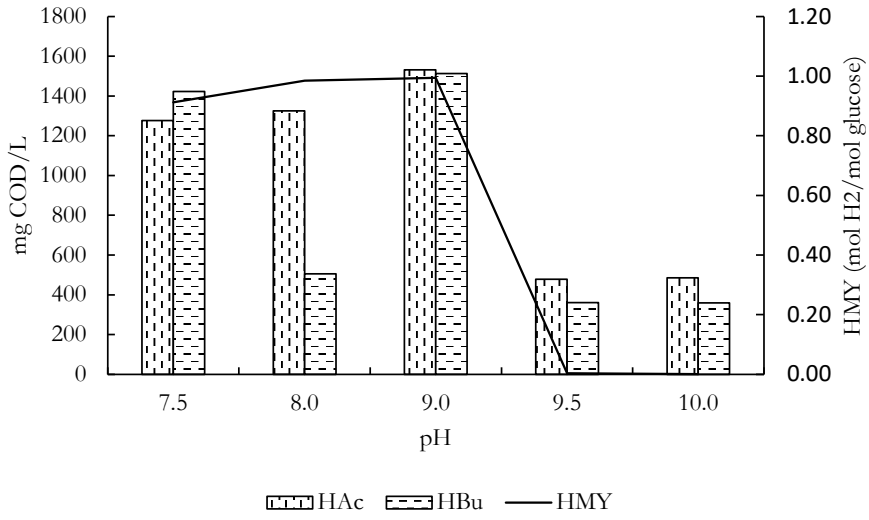

Figure 2. Volatile Fatty Acid composition at different pH conditions (HAc = acetic acid, HBu = butyric acid, HMY = hydrogen molar yield). Temperature 37°C, 26% NaCl. Volume was measured as STP.

Table 2. Comparison for hydrogen yield of fermentation by halophilic bacteria.

Carbon source	Inoculum	NaCl	T(°C)	pH	HMY	Reference
Cellobiose	*Halanaerobium hydrogeniformans*	7%	33	11	2.3±0.2 mol H_2/ mol cellobiose	[9]
Glucose	*H. hydrogeniformans*	7%	33	11	2.42 mmol H_2/ mmol glucose	[9]
Glucose	*H. salinarius*	14-15%	NI	NI	2 mM/4.17 mM	[10]
Glucose	*H. chitinovorans*	12%	37	5	144.5 μmol H_2/ 5 mL 0.5% glucose	[11]
Glycerol	*H. saccharolyticum senegalensis*	150 g/L	37	7	1.6 mol H_2/ mol glycerol	[12]
Glycerol	*H. saccharolyticum saccharolyticum*	150 g/L	37	7.4	0.6 mol H_2/ mol glycerol	[12]
Glucose	Mixed culture from salt factory wastewater	75 g/L	35	8	0.9 ± 0.02 mol H_2/mol glucose	[21]
Glucose	Mixed culture from a salt pan	26%	35	9	1.45 mol H_2/ mol glucose	this study

T=temperature, HMY=hydrogen molar yield, NI= no information

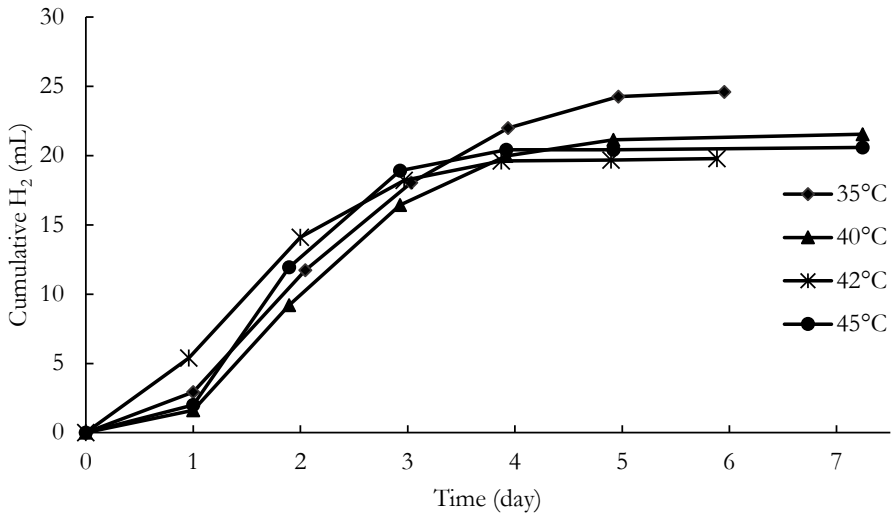

Figure 3. Cumulative biohydrogen production at different temperature conditions (pH 9, NaCl 26%. Volume was measured as STP).

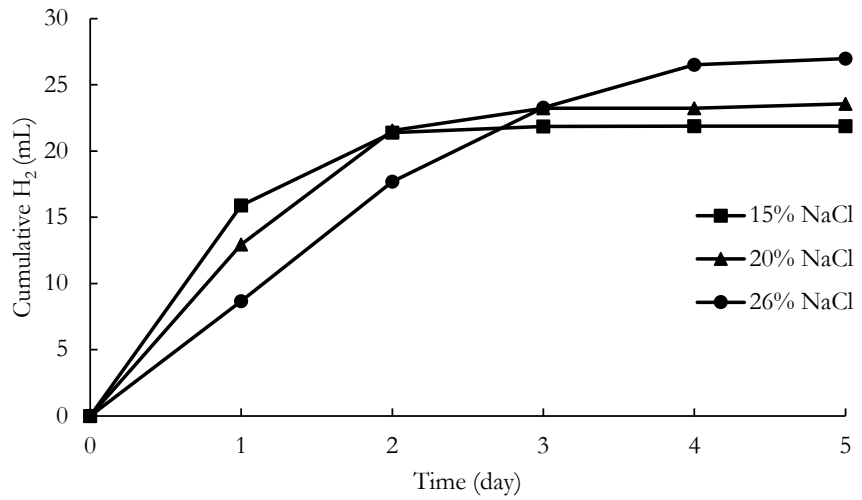

Figure 4. Cumulative biohydrogen production at different NaCl concentrations (Temperature 35°C, pH 9. Volume was measured as STP).

of high salt concentrations by maintaining a high intracellular KCl concentration [24].

3.2 Carbohydrate Fermentation Test

The acclimatized seed microorganisms from Samut Sakhon salt pan were capable of fermenting various types of hexoses, pentoses, dissacharides, and starch, as well as glycerol (Table 3).

Lignocellulosic-derived sugars include glucose, galactose, mannose, xylose, arabinose, fucose, and rhamnose [25,26]. The fermentation test results indicated that the seed microorganisms were able to ferment these types of sugars. The microorganisms' ability to produce a copious amount of biogas from D-fructose suggested a potential ability to produce biohydrogen from food waste.

3.3 Identification of Hydrogen Producer of the Acclimatized Mixed Culture from Samut Sakhon, Thailand

The strongest band in DGGE analysis were band 1 and 2 (Figure 5). Excised bands 1 and 2 showed the same sequencing results. For the V3 region of 16s rRNA sequences, the NCBI's MegaBLAST confirmed 100% similarity of these bands with *H. fermentans* strain R-9. The phylogenetic tree showing the relationship between the sequence of the excised bands and other *Halanaerobium* species is shown in Figure 6. Colony PCR of 16s rRNA, which was later performed to confirm these results, showed 99.3% similarity with *H. fermentans* strain R-9 using NCBI's MegaBLAST and 99.36% similarity using EzBioCloud. The resulting sequence of colony *H. fermentans* strain B4 (1424 bp) has been submitted to the GenBank data base (Accession number MN133965).

H. fermentans was first isolated from salted puffer fish ovaries in Japan [18,27]. This gram-negative, motile, strictly anaerobic bacteria species was also reported to be present in other salted food products from other countries including *yegyo ngapi* from Myanmar [28], *pla-ra* from Thailand, *pa-daek* from Laos [29], salted and fermented seafood from Korea [30]. Fermentation products of *H. fermentans* include acetate, formate, lactate, ethanol, H_2, and CO_2 [18].

Although other bacteria in the mixed culture could not be identified yet in this study, these bacteria played a substantial role in fermentation. The mixed culture was capable of fermenting L-arabinose in repeated tests; however, pure cultures of *H. fermentans* did not have this ability in our study. This difference in carbohydrate utilization ability was also observed for L-rhamnose, starch, and glycerol, which were fermentable by the mixed culture, but showed negative results in the report by Kobayashi *et al.* [18]. Table 3 shows a comparison of carbohydrate fermentation ability between mixed culture from this study and pure *H. fermentans* strain R-9 culture. The VFA measurements also showed that butyric acid, a metabolite which is reported not to be produced by *H. fermentans* [18], was produced by the mixed culture (Figure 2). This phenomenon demonstrated the benefits of mixed culture utilization in the fermentation of waste-derived carbohydrates, highlighting the versatility of utilizing different carbohydrate sources.

H. fermentans may have the potential to provide a valuable resource for metabolic engineering in biohydrogen production from high-salinity waste, such as pretreated lignocellulosic waste. However, genome examination will be required to identify the specific pathways that can be modified to maximize biohydrogen production yields. To date, the complete genome of this bacterium has not yet been published, unlike those of *H. saccharolyticum*, *H. praevalens*, and *H. hydrogeniformans*. The ferredoxin hydrogenase pathway is the most common hydrogen-producing exchange pathway in strict anaerobes. However, the genome of *H. hydrogeniformans* has been reported to lack this enzyme [31].

Table 3. Carbohydrate fermentation test of acclimatized seed microorganisms from Samut Sakhon salt pan compared to the pure culture of *Halanaerobium fermentans* R-9.

Carbon sources	Samut Sakhon acclimatized seed inoculum		*H. fermentans* R-9 [18]
	Fermentation test result	Biogas production (mL)	
D-glucose	+	1.27 ± 0.55	+
D-galactose	+	0.27 ± 0.06	+
D-mannose	+	1.57 ± 0.21	+
L-fucose	+	0.95 ± 0.15	NI
L-rhamnose	+	0.10 ± 0.17	-
D-xylose	+	0.30 ± 0.17	+
L-arabinose	+	1.04 ± 0.37	-
D-maltose	+	0.30 ± 0.00	+
Sucrose	+	1.00 ± 0.00	+
D-fructose	+	2.13 ± 0.32	+
D-cellobiose	+	0.27 ± 0.15	+
Trehalose	+	0.40 ± 0.17	NI
Lactose	+	0.20 ± 0.14	+
Starch	+	1.03 ± 0.06	-
α-cellulose	-	0 ± 0.00	NI
Glycerol	+	0.07 ± 0.06	-

NI: no information. Fermentation period: 6-19 days. Inoculum: 3 drops. Volume was not measured as STP.

Figure 5. PCR-DGGE result of V3 region of the 16S rRNA from Samut Sakhon acclimatized mixed culture. The brightest bands (1 and 2) were excised, sequenced, and identified to GenBank's data base. NCBI's MegaBLAST confirmed 100% similarity of these bands (194 bp) with *H. fermentans* strain R-9.

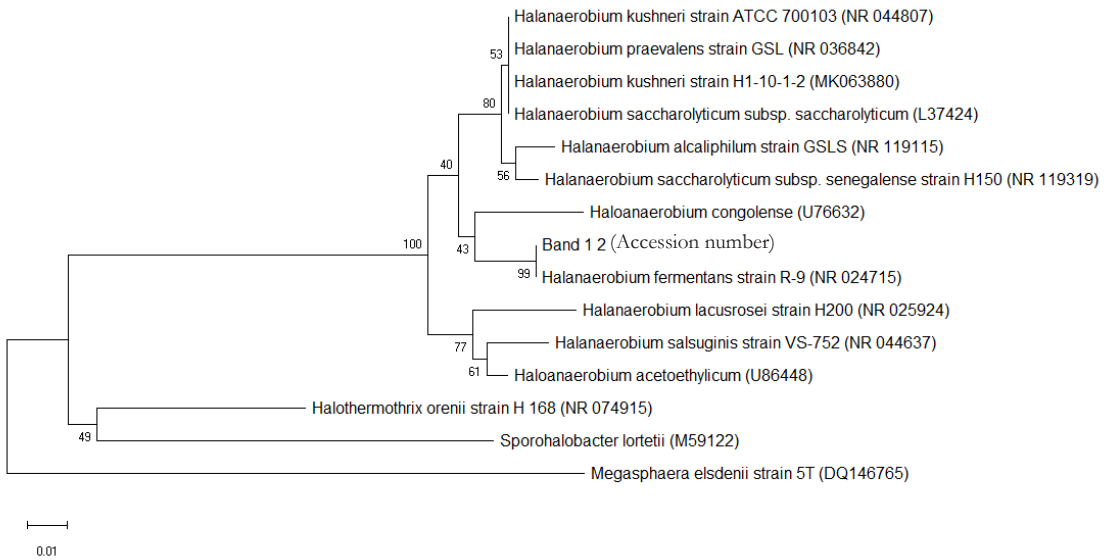

Figure 6. Phylogenetic tree showing the relationships between the excised bands and other related species based on the V3 region of the 16S rRNA gene. The tree was based on Jukes-Cantor distance and constructed using the Neighbor-Joining method with 1,000 bootstraps using Mega X. The scale bar represents 0.01 substitutions per nucleotide position. *Megasphaera elsdenii* was used to root the tree. The percentage of replicate trees clustered together in the boostrap test are indicated at the nodes. Reference sequences in the dendrogram were obtained from the NCBI's GenBank.

4. Conclusions

The optimum conditions for biohydrogen production by extremely halophilic bacteria from Samut Sakhon salt pan in Thailand was determined. The highest obtained HMY was 1.45 mol H_2/mol glucose, under optimum conditions of 26% NaCl (351 g/L), 35°C, and pH 9. The acclimatized bacteria were able to ferment various types of lignocellulosic-derived sugars. The predominant hydrogen producer among the mixed culture was *H. fermentans*, although other unidentified bacteria also played a substantial role in the fermentation process. Future studies are needed to study biohydrogen production from lignocellulosic and food waste biomasses from the acclimatized bacteria. Moreover, additional investigations of other community members in the mixed culture should also be performed.

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