AIMS Energy, 6(5): 846–865.
DOI: 10.3934/energy.2018.5.846
Received: 11 August 2018
Accepted: 11 October 2018
Published: 18 October 2018

http://www.aimspress.com/journal/energy

Research article

Hydrogen production by newly isolated *Clostridium* species from cow rumen in pure- and co-cultures on a broad range of carbon sources

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Abstract: Three novel hydrogen-generating strains, ST1, ST4, and ST5, were isolated from the rumen of cow in Vietnam, and respectively identified as *Clostridium beijerinckii* ST1, *Clostridium bifermentans* ST4, and *Clostridium butyricum* ST5, based on 16S rDNA gene sequence analysis and physiobiochemical characteristics. The dark fermentative hydrogen production of these isolated *Clostridium* strains was performed and characterized in both pure- and co-cultures from various carbon sources including sucrose, glucose, lactose, xylose, molasses, cassava stumps, and rice distillers wet grains with soluble. The highest hydrogen production was achieved from a co-culture with three *Clostridium* strains. To optimize the operational conditions of temperature, time, and substrate concentration for the high-level production of hydrogen, response surface methodology in a Box-Behnken design was used. The results revealed a maximum hydrogen production of 1.13 ± 0.015 L H_2/L medium by the three-strain co-culture under the following fermentation conditions: 11.63 g/L sucrose, 36.1 °C, in 51.13 h.

Keywords: hydrogen production; *Clostridium*; co-culture; cow rumen bacteria; response surface methodology; food industry wastes

1. Introduction

Hydrogen (H_2) is a renewable energy source and a promising alternative to conventional fossil fuels because it is capable of eliminating most of the problems caused by widespread fossil fuel use. Several processes are known to produce H_2, including the electrolysis of water, thermocatalytic reformation of hydrogen-rich organic compounds, as well as geological and biological processes [1]. Biological production of H_2 using microorganisms via photo- and dark-fermentation is now an exciting area of technology development. This approach offers much promise because it requires a lower energy supply and provides other benefits compared with chemical-physical technologies to produce H_2 from a variety of renewable resources [2,3]. Specifically, via fermentation processes, H_2 can be generated directly from renewable substrates, such as sugars, biomass, or even organic residues, such as agricultural and food-industry waste and wastewater, in high concentrations [4].

Dark fermentative H_2 production is a ubiquitous phenomenon under anoxic or anaerobic conditions [5]. Many bacteria use the reduction of protons to H_2 via hydrogenases as a way to oxidize the reduced carriers during the fermentation process [6]. The theoretical yield of H_2 from glucose fermentation could be estimated from its known metabolic pathway, offering a maximum yield of 4 mol H_2/mol glucose if all of the substrates were converted to acetic acid as per the chemical reaction [7].

Dark fermentative H_2 production has been studied for a large group of pure bacterial cultures, including species of *Enterobacter*, *Bacillus*, *Ethanoligenens*, and *Clostridium* [2]. In taking advantage of their high-yielding H_2 production, many strains of *Clostridium* have been isolated and studied both as pure strains and in co-cultures for realizing efficient H_2 production [8–15].

In the cow, the rumen is the largest stomach compartment, which harbors a complex community populated by microorganisms such as bacteria, archaea, protozoa, and fungi [16]. The rumen microbes can ferment plant fibers (e.g., hemicellulose and cellulose), starch, sugar, and protein to produce volatile fatty acids (e.g., acetate, propionate, butyrate, and lactate), microbial protein, and gases, such as CO_2, H_2, and CH_4 [17]. Many microorganisms produce H_2 in the rumen; however, methane bacterial populations also grow in the rumen, converting the CO_2 and H_2 into CH_4 [18]. Consequently, very little of the total rumen gases consists of H_2 Nevertheless, cow rumen fluid has been investigated and studied for its H_2 production from lignocellulose or cellulose under the inhibition of rumen methanogenesis by heat or an acid pre-treatment [18,19]. These studies, however, focused solely on the H_2 production of the rumen fluid enrichment culture as a mixed system composed of dominant *Clostridium* species (based on their 16S rDNA sequence and PCR-DGGE profiles analysis).

To specify the promising *Clostridium* strains for H_2 production, this study aimed to isolate, characterize, and identify the H_2-producing *Clostridium* spp. in cow rumen. Among the isolated *Clostridium* strains, three new ones (ST1, ST4, and ST5) were designated as C. *beijerinckii* ST1, C. *bifermentans* ST4, and C. *butyricum* ST5, based on their 16S rDNA gene sequence analysis and physiobiochemcal characteristics. Sucrose, glucose, lactose, xylose, and molasses were used as model carbon sources, to evaluate the H_2-production ability of these strains from different culture modes: three pure *Clostridium* cultures (ST1, ST4, and ST5) and four different co-cultures which consisted of (1) ST1 and ST4, (2) ST1 and ST5, (3) ST4 and ST5, and (4) mixing all three strains.

In the other hands, the food industry wastes such as cassava tuber wastes (cassava stumps) and a by-product of ethanol industry (distillers wet grains with soluble) are abundant, cheap, renewable,

and rich nutrition of carbon source in tropical countries including Vietnam. Therefore, such wastes can be exciting and promising substrates for biological H_2 production by *Clostridium* sp. and they can give a significant benefit for bioenergy industrial in economic. However, until now, there has been no research on H_2 production from cassava stumps and distillers wet grains by *Clostridium* sp. yet. Hence, we use cassava stumps and distillers wet grains with soluble from cassava flour and ethanol manufacturing processes as the main substrate for H_2 production by the isolated *Clostridium* strains in this study.

Furthermore, response surface methodology using the Box-Behnken design and the software Design-Expert v7.1.5 was applied to optimize the operational conditions—temperature, time, and substrate concentration—to determine the most effective H_2 production by the three-strain co-culture.

2. Materials and methods

2.1. *Isolation and identification of H_2-producing strains*

One cow rumen (Quoc Oai, Hanoi, Vietnam) was sampled and pretreated by heating at 90 °C for 20 min to obtain the isolated strains. A peptone–yeast extract (PY) medium—10 g of glucose (Purity of 99%, Biobasic, Canada), 10 g of peptone (Biobasic, Canada); 10 g of yeast extract (Biobasic, Canada); 1 mg of resazurine (Purity of 99.9%, Sigma, USA), and 10 mL of a solution of salts [20]—was used to culture and screen the H_2-producing bacterial strains. The initial pH of the medium was adjusted to 6.5. The experiments were performed in 15 mL glass bottles using a 10 mL working volume. After inoculation, the headspace of the bottles was flushed with nitrogen gas for 15 min, to ensure an anaerobic environment in them. The bottles were kept in a constant temperature-controlled incubator at 37 °C, with agitation at 120 rpm for 48 h. This process was replicated three times, and then inocula were spread onto PY-agar medium in an anaerobic culture box to obtain single colonies producing H_2. Then, these obtained strains were transferred onto fresh medium and cultured at 37 °C for 48 h.

The isolated strains that yielded high H_2 production were selected for identification by standard methods [20] and by 16S rDNA gene sequence analysis. Total genomic DNA was extracted from each strain by using the Magpure Bacteria DNA Kit (ANABIO Research and Development, Hanoi, Vietnam). The 16S rDNA gene sequence was amplified by conventional PCR using two primers, 27F (5'-AGAGTTTGATAMTGGCTCAG-3') and 1527R (5'-AAAGGAGGTGATCCAGCC-3'). PCR reactions were performed in a DNA thermal cycler, for which the reaction conditions were set as follows: 96 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 62 °C for 45 s, 72 °C for 90 s, with a final extension at 72 °C for 5 min. The PCR products were purified by an Anapure PCR Product Kit (ANABIO Research & Development, Hanoi, Vietnam) and sequenced by the 1st Base company (Singapore). The 16S rDNA sequence of these strains was analyzed with BioEdit v7.2 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned against existing sequences in the GenBank database by using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed in CLUSTAL v1.8 based on the neighbor-joining method with 1000 bootstrap replicates.

2.2. Physiobiochemical characteristics

Pure isolated strains were tested for their morphology, physiology, and biochemistry properties. Although this approach is unlikely to be entirely accurate, it represents the first step taken in microbial classification research. The methods used for physiobiochemical characterization of isolated strain followed the standard protocol that has been conventionally used in bacterial systematic [20].

2.3. Enzymatic activity of the isolated strains

To evaluate the capacity of the isolated *Clostridium* strains for biohydrogen fermentation from different organic substrates, the enzymatic activities (amylase, protease and cellulase) were investigated via the diffusion method on agar plates. The *Clostridium* spp. were cultured separately on PY medium, which was added 10% (w/v) soluble starch (Biobasic, Canada), casein (Sigma, USA), and carboxymethyl cellulose (CMC) (Sigma USA), under conditions of 37 °C, with a shaking rate of 200 rpm, for 24–48 h. Bacterial suspensions were centrifuged at 7000 rpm, discarding the biomass while keeping the supernatant. We used 1000-μL tips to perforate Petri dishes containing agar (with 1% soluble starch, casein, and CMC added). After dropping the bacterial suspension into the agar perforations, the agar Petri dishes were kept at 4 °C for 3–6 h, and then incubated at 37 °C for 24–48 h. Two petri dishes with 1% soluble starch and CMC were then stained with a Lugol-5% solution (Sigma, USA).

2.4. Pure cultures and co-cultures

The cultures were grown in 15 mL serum bottles sealed with a rubber stopper and an aluminum stopper containing 10 mL of PY medium including 10 g carbon source at 37 °C and pH 6.5 with 10% (v/v) inoculation, which released OD_{600} of 0.1 for the primary medium and shaken at 220 rpm. The headspace of the serum bottle was sparged under an atmosphere of 99.999% N_2 to exclude oxygen and thereby create a stable anaerobic environment completely. To select the best suitable carbon source for fermentative H_2 production, each candidate carbon source such as glucose, sucrose, lactose, xylose, molasses, cassava stumps, and distillers wet grains with soluble was alternately tested at 10 g/L at final concentration.

2.5. Sampling and analyses

Growth was monitored by optical density (OD_{600}) with the sterile medium as the control. Hydrogen gas in the headspace was sampled with a gas-tight syringe (100 μL injection volume, Hamilton, USA) and determined by Shimadzu GC-8A gas chromatograph equipped with a thermal conductivity detector (GC/TCD) and two columns (Alltech, USA). Accumulative H_2 production was calculated by using the standard curve as described in the Figure S2 in the Supplementary data. Operating conditions were set: the carrier gas was nitrogen, under a pressure of 80 kPa; temperature of the column was fixed at 145 °C, and the temperature of both the injector and detector was 150 °C. The glucose concentration was determined with the dinitrosalicylic acid (DNS) colorimetric method as described by Miller [21]. All analyses were run in triplicate.

2.6. Experimental design for response surface methodology

To optimize the key parameters for enhancing the H_2 yield of the dark fermentative process, a 3^k Box-Behnken Design was employed using the software Design-Expert v7.1.5. For the statistical calculations, the relationship between the coded values and real values is described as follows:

$$X_i = (A_i - A_o)/\Delta A \quad (1)$$

where X_i codes the variable value, A_i is the real variable value, A_o is the value of A_i at the center-point; and ΔA is the step-change of the variable. Table S1 summarizes the levels of the variables and the experimental design containing the 17 trials; the response values were expressed as the mean of triplicates. The second-order polynomial coefficients were also calculated and analyzed in the Design Expert software (v7.1.5) statistical package. The H_2 yield was selected as the response variable, while the carbon source concentration (X_1), fermentation time (X_2), and fermentation temperature (X_3) were selected as the three independent variables. An analysis of variance (ANOVA) was carried out to validate the statistical results and the model equation.

3. Results and discussion

3.1. Isolation and biochemical characterization of strains showing their potentials

3.1.1. Isolation and identification of strains

Eight strains were isolated from the rumen of cow (Table S2, Supplementary Data). Among the isolates, the strains ST1, ST4, and ST5 respectively produced the highest H_2 production (mean ± SD) of 459.25 ± 5.31, 568.05 ± 8.27, and 439.19 ± 8.42 mL/L medium, from the PY standard medium containing glucose as the carbon source. These strains were identified by standard biochemical analysis [20] for specific characteristics, as shown in Table S3 and Figure 1. The results indicated that the isolated strains ST1, ST4, and ST5 possessed general features of the *Clostridium* genus, such as Gram-positive staining, negative catalase functioning, spore formation, etc.

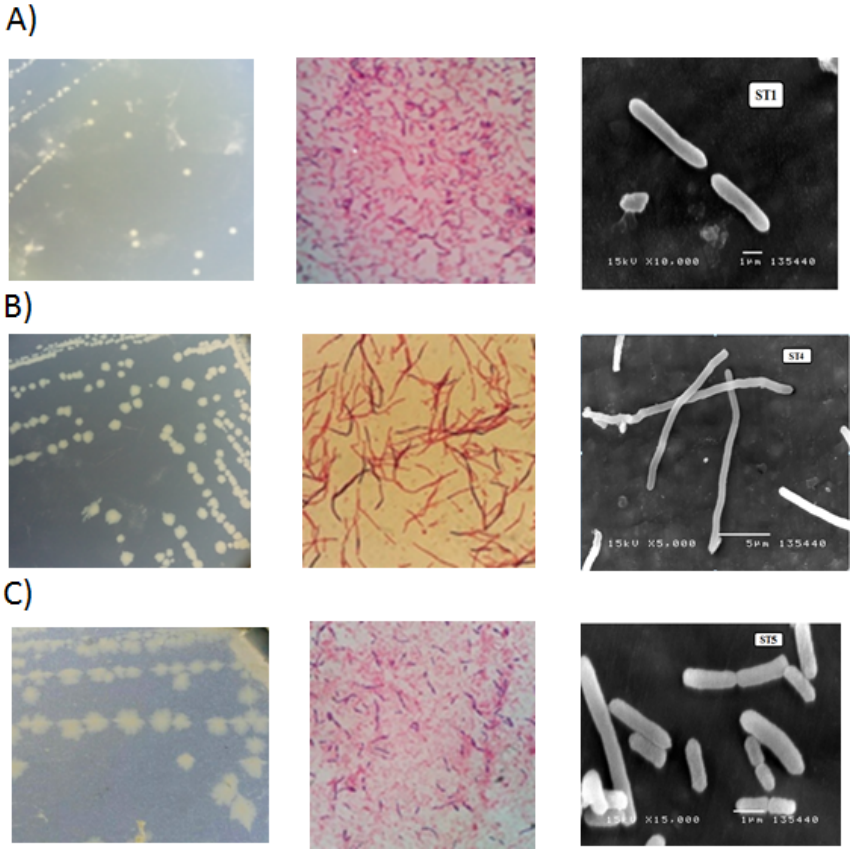

Figure 1. Morphology of isolated strain ST1 (A), ST4 (B), and ST5 (C). Colony, Gram staining, and Scanning Electron Microscope (SEM) image of *Clostridium* strains images respectively indicated in the left, middle, and right.

The colony of strain ST1 was milk-white in color, circular, opaque, and had a diameter of c. 1 mm after culturing on an agar plate at 37 °C for 48 h. Morphologically, cells of ST1 in the PYG culture appeared as straight rods with rounded ends, 0.5–1.7 × 1.7–8.0 μm (Figure 1A, right). The colony of strain ST4 was circular with irregular margins, flat or raised, lobate or scalloped, translucent or opaque, gray, shiny and smooth, and had a diameter of c. 0.5–4.0 mm after culturing on an agar plate at 37 °C for 48 h. Morphologically, cells of ST4 in the PYG culture appeared as straight rods, 0.6–1.9 × 1.6–11.0 μm (Figure 1B, right). The colony of strain ST5 was convex, translucent, gray-white, lobate or slightly scalloped, and had a diameter of c. 4–6 mm after cultivation on an agar plate at 37 °C for 48 hours. Its cells in the PYG culture appeared as straight rods with rounded ends, 1–1.7 × 1.7–8.0 μm, occurring singly or in pairs (Figure 1C, right).

For further confirmation, the 16S rDNA sequences of these strains were analyzed and aligned against existing sequences in the NCBI GenBank database using the BLAST program. The 16S rDNA gene sequence was directly amplified from the genomic DNA obtained from each strain. The results indicated a 100%, 99%, and 100% identification with *Clostridium beijerinckii* NCIMB 8052-CP000721 (NCBI No. MF136817), *Clostridium bifermentans* ATCC638 (NCBI No. MF125286), and *Clostridium butyricum* DSM10702 (NCBI No. MF125285) for the isolated strain ST1, ST4, and ST5, respectively (Figure 2). Thus, these three isolated strains were designated new strains of the

species *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5, as determined by the 16S rDNA gene analysis and their physicochemical characteristics.

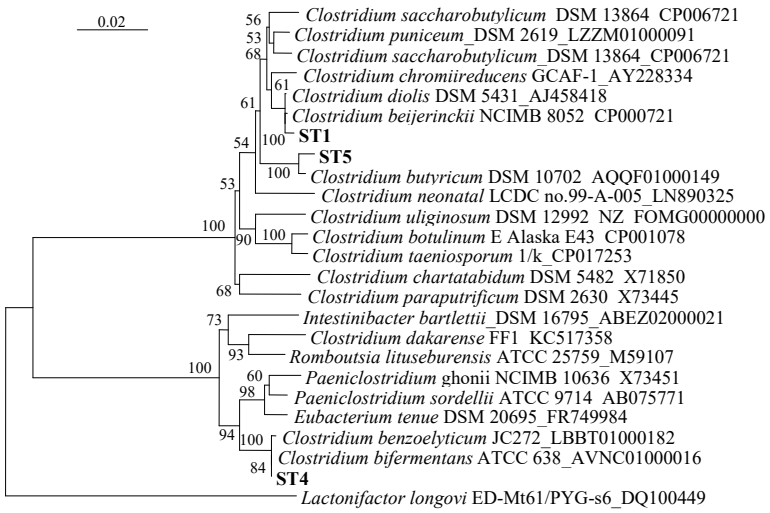

Figure 2. Phylogenetic tree shows the relationship between strain ST1, ST4, and ST5 and related species based on 16S rDNA gene sequence analysis.

3.1.2. Enzymatic activity of isolated *Clostridium* strains

The three newly isolated strains *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5 produced protease (Figure S1A, Supplementary Data). A light ring surrounding the perforation appeared in the Petri dishes containing agar (with 1% casein added) after incubating at 37 °C for 24 h. This result indicated that casein (a protein in milk) was hydrolyzed by protease. To get a good visualization, the Lugol reagent was utilized to get a good visualization. The ring size of *C. bifermentans* ST4 indicated that its ability to decompose protease was superior to the other two strains.

The activity of amylase in the *Clostridium* isolated strains was also confirmed (Figure S1B, Supplementary Data). The part of the agar medium not hydrolyzed by amylase became blue-violet when stained with the Lugol reagent. The main component of the Lugol reagent is potassium iodide (KI). Thus, a reaction occurred between soluble starch (the substrate) and KI to create the characteristic blue-violet color. A light ring around the perforation revealed that three strains *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5 could all produce amylase. Their ring sizes indicated that the enzyme decomposition potential of these isolated strains was quite high (ring diameters of 10–15 mm).

As Figure S1C shows, the three isolated strains all produced cellulase. A light ring around the perforation appeared after staining with Lugol reagent. Comparing the ring sizes indicated that the ability to decompose cellulose was stronger in *C. bifermentans* ST4 than in the other two strains (Figure S1C, middle; Supplementary Data).

Based on the enzymatic activity of *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5 (Figure S1, Supplementary Data), the organic wastes and agriculture residues, are available for H_2 production via these three strains.

3.1.3. BioH_2 production from the model substrates

To evaluate the capacity of *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5, various substrates, including monosaccharides (glucose and xylose), disaccharide (sucrose and lactose), oligosaccharide (molasses) from sugar manufacturing, and complex matrix substrates from food industry wastes such as cassava stump and distillers wet grains with solubles (DWGS), were used as different carbon sources for H_2 production in a series of batch cultures that tested the capacity of pure cultures and co-cultures.

As Figure 3A shows, of the five substrates examined, H_2 production was observed by using pure *Clostridium* spp. cultures with four main types of carbon sources: glucose, sucrose, lactose, and xylose. However, the amount of H_2 production varied depending on the kind of substrate and the particular strains used. For glucose, the highest H_2 production (mean ± SD) of 732.15 ± 11.8 mL H_2/L (1.36 mol H_2/mol glucoseconsumed) was obtained using *C. beijerinckii* ST1 (Table S4, Supplementary Data), for which the obtained H_2 percentage was 51.7% and the OD_{600} value was 1.794 ± 0.035. Glucose is also a suitable substrate for *C. bifermentas* ST4: this strain had an H_2 production of 675.37 ± 8.32 mL H_2/L or H_2 yield of 2.47 mol H_2/mol glucoseconsumed and an OD_{600} value of 1.489 ± 0.045 (Table S3, Supplementary Data). The H_2 production obtained using *C. butyricum* ST5, with glucose as substrate, was lower, at 548.4 ± 3.74 mL H_2/L or H_2 yield of 0.7 mol H_2/mol glucoseconsumed (Table S4, Supplementary Data). Glucose, however, is one of the most suitable carbon sources because it is directly involved in the digestion process, which facilitates the growth of bacteria and, by extension, their H_2 yield. These results suggest that glucose is indeed a suitable carbon source for H_2 production, which agrees with prior research [22]. For example, Lin et al. reported H_2 yields of 2.81 and 1.8 mol H_2/mol glucoseconsumed when using *C. beijerinckii* L9 and *C. butyricum* ATCC19398, respectively [22]. In comparison with the previous studies (Table 1), *C. beijerinckii* ST1 and *C. bifermentas* ST4 indicate relatively strong potential for H_2 production.

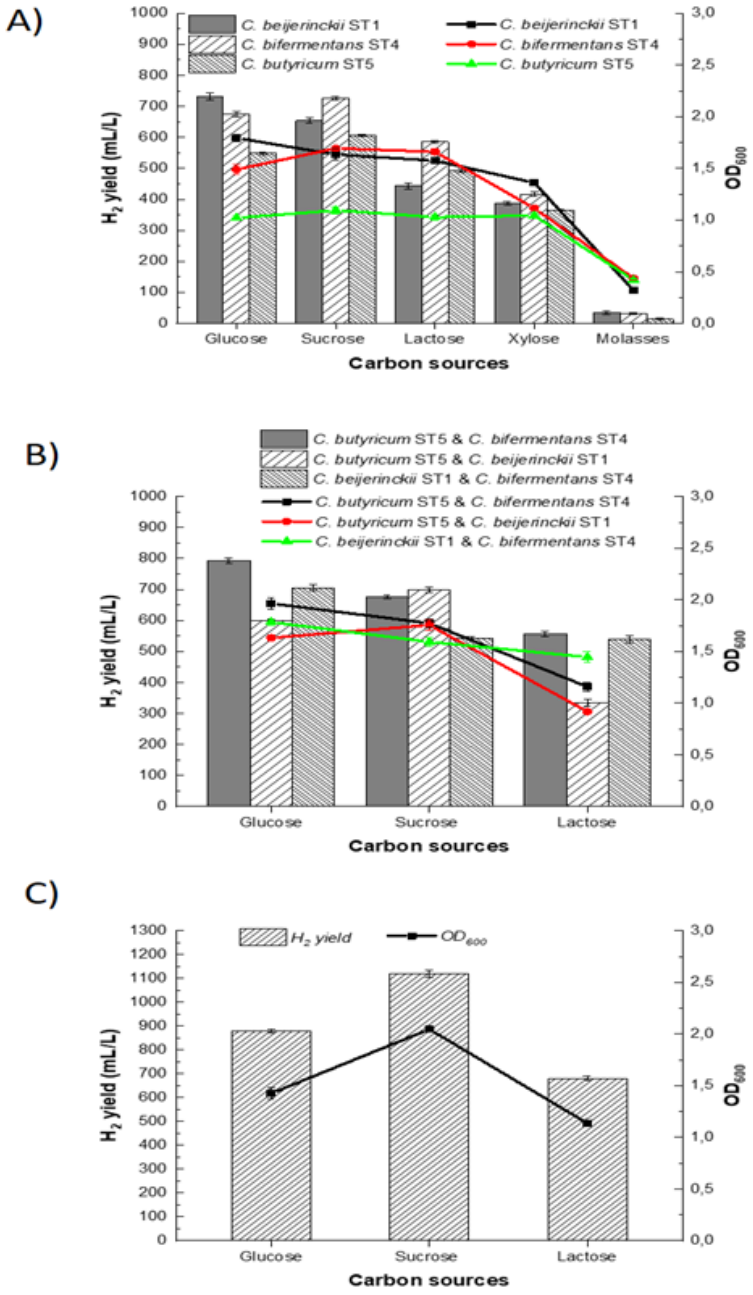

Figure 3. Growth and H_2 production from different carbon sources by using pure *Clostridium* cultures (A), co-cultures of two *Clostridium* sp. (B), and co-cultures of three isolated strains including *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5 (C). Bar charts indicate the H_2 production. The Line charts show the growth of *Clostridium* strains based on absorbance of OD at a wavelength of 600 nm.

Table 1. H_2 production from isolated *Clostridium* sp. in this study compared to different *Clostridium* species in the previous reports.

Strains	Glucose (g/L)	Temp. (°C)	pH	H_2 Yield (mol/mol $glucose_{consumed}$)	Ref.
C. butyricum IFO 3847	1	37	7.0	0.9	[24]
C. butyricum IFO 3847	9	37	7.0	1.26	[25]
C. butyricum IAM 19002	9	37	7.0	1.04	[25]
C. butyricum IAM 19003	9	37	7.0	1.2	[25]
C. butyricum CWBI1009	1–10	30–37	5.2–8.0	0.23–2.4	[26–31]
C. butyricum A1	10	37	6.5	1.9	[32]
C. beijerinckii RZF 1108	5–9	37	5.0–7.0	0.53–1.75	[12]
C. beijerinckii RZF 1108	9	35	7.0	1.97	[12]
C. beijerinckii Fanp3	10	36	6.5	2.52	[10]
C. beijerinckii NCIMB8052	5	37	7.0	0.6–2.1	[33]
Clostridium sp. W1	–	35	–	0.51	[34]
Clostridium sp. AK15	–	60	6.0	0.8	[35]
C. tyrobutyricum ATCC 25755	30	37	5.7	2.0	[36]
C. perfringens strain JJC	5	37	6.0	4.68	[14]
C. bifermentans strain WYM	5	37	6.0	3.29	[14]
Clostridium sp. strain Ade.TY	5	37	6.0	2.87	[14]
C. beijerinckii ST1	10	37	6.5	1.36	In this study (Table S4, Supplementary data)
C. bifermentans ST4	10	37	6.5	2.47	
*C. butyricum*ST5	10	37	6.5	0.7	
C. beijerinckii NBRC	10	37	6.5	1.07	

Sucrose is also considered a suitable substrate for H_2 production. Previous research had shown that H_2 was relatively high when sucrose served as a carbon source. Chen et al. reported an H_2 yield of 2.78 mol H_2/mol of sucrose from *C. butyricum* CGS5 [37]. In our study, the highest H_2 production of 725.8 ± 5.6 mL/L was obtained using C. bifermentans ST4, accounting for 55.3% of the total gas production, with an OD_{600} value of 1.694 ± 0.014. Next one was *C. beijerinckii* ST1, which obtained 654 ± 8.12 mL/L and had an OD_{600} value of 1.634 ± 0.057. The H_2 production with *C. butyricum* ST5 (606.4 ± 2.08 mL/L) was lower than that with either *C. beijerinckii* ST1 and *C. bifermentans* ST4. Although H_2 production from lactose was lower than that from glucose and sucrose, it remained a suitable substrate for *C. bifermentans* ST4, which had an H_2 production of 585.5 ± 4.9 mL/L on this substrate (Table S5, Supplementary Data). Figure 3A indicates that xylose also was a substrate for H_2 production by fermentation with *C. beijerinckii* ST1, *C. bifermentans* ST4, *C. butyricum* ST5; their corresponding H_2 production were 387.8 ± 5.3, 417 ± 6, and 364.8 ± 1.7 mL/L (Table S5, Supplementary Data). Molasses was not a suitable substrate for H_2 production. Previous study reported that the molasses contains several phenolic compounds derived from sugarcane such as dehydrodiconiferylalcohol-9'-O-beta-D-glucopyranoside and isoorientin-7, 3'-O-dimethyl ether, which possesses antibacterial activity against bacteria [38]. As a consequence, the highest H_2 yield achieved was only 33.8 ± 5.3 mL/L, for *C. beijerinckii* ST1 (Figure 3A, Table S5, Supplementary Data).

3.2. The use of strains in co-cultures with different substrates

3.2.1. H_2 production from the pure substrates

Based on the pure culture results, sucrose, glucose, and lactose as the carbon sources were chosen to investigate H_2 production by co-cultures at 37 °C for 48 h. Figure 3B shows that the highest H_2 production was achieved by the *C. butyricum* ST5 and *C. bifermentans* ST4 co-culture using glucose, for which the H_2 percentage was 54.7% and the H_2 production was 793.8 ± 8.1 mL/L (Table S5, Supplementary Data). This value was 1.17- to 1.44-fold that of either pure culture and greater than the highest H_2 production obtained with *C. beijerinckii* ST1 (732.2 ± 11.8 mL/L) (Table S5, Supplementary Data). In addition, the OD_{600} value also increased from 1.021 ± 0.023 to 1.965 ± 0.056. Collectively, these results confirm that glucose is a suitable substrate for co-cultures of *C. butyricum* ST5 and *C. bifermentans* ST4. However, the hydrogen yield of the *C. butyricum* ST5 and *C. beijerinckii* ST1 co-culture was <18% that of *C. beijerinckii* ST1, while the OD value decreased from 1.794 ± 0.035 to 1.632 ± 0.031. A similar result was obtained for the *C. beijerinckii* ST1 and *C. bifermentans* ST4 co-culture, which points to resource competition among microorganisms as a possible explanation.

For sucrose, the highest H_2 production was obtained for the co-culture of *C. butyricum* ST5 and *C. beijerinckii* ST1, which amounted to 699.8 ± 8.8 mL/L, and accounted for 55.1% of the total volume of biogas produced. This value is 1.15- to 1.07-fold greater than what their pure cultures achieved, with an increase in OD_{600} from 1.094 ± 0.034 to 1.765 ± 0.054. However, this particular *Clostridium* co-culture still had a lower H_2 production than that of *C. bifermentans* ST4 (725.8 ± 5.6 mL H_2/L, OD_{600} = 1.694 ± 0.014). An H_2 production of 676.4 ± 5.24 mL/L and an OD_{600} value of 1.774 ± 0.043 were obtained for the co-culture of *C. butyricum* ST5 and *C. bifermentans* ST4. Although this OD_{600} value is higher, the H_2 production is lower; this suggests that, when using sucrose as a substrate, microorganisms would likely grow better in co-cultures of ST5 and ST4, though in the decomposition process acetic, butyric, and ethanol byproducts were generated. The results for the *C. beijerinckii* ST1 and *C. bifermentans* ST4 co-culture also indicated that both H_2 production and OD_{600} values decreased, which may be explained by competition between the *Clostridium* strains.

Besides using co-cultures of two *Clostridium* species, many studies have reported on the mixing of *Clostridium* with other species to produce H_2. For example, Ding *et al.* obtained a maximum H_2 yield of 3.47 mol H_2/mol $glucose_{consumed}$ by using a co-culture of *C. butyricum* and immobilized *Rhodopseudomonas faecalis* RLD-53 [39]. Geng et al. obtained an H_2 yield of 1387 mL/L when they combined *C. thermopalmarium* and *C. thermocellum* [40]. This is consistent with that of Liu et al., who obtained an H_2 yield of 1232 mL/L with co-cultures of *C. thermocellum* JN4 and *C. thermosaccharolyticum* GD17 [41]. Finally, from a co-culture of *Bacillus themoamylovorans* I and *C. beijerinckii* L9, Chang et al. obtained an H_2 yield of 1145 mL/L [42].

Figure 3C depicted the growth and H_2 production vitality when the three isolated strains were evenly mixed (1:1:1) to investigate their H_2 production at 37 °C within 48 h. In this experiment, the maximum H_2 production of 1120.0 ± 14 mL H_2/L (Figure 3C, Table S5, Supplementary Data) was obtained when using sucrose as a substrate, for which the percentage of H_2 gas was 61.8% of the total biogas produced, and the OD value was 2.046 ± 0.027. This result clearly shows that sucrose is the most suitable carbon source for both bacterial growth and H_2 production from co-culture mixing *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5. A plausible explanation is that, when the organisms are present in combination, genes encoding enzymes hydrolyzing sucrose to

glucose and fructose are strongly expressed, so that sucrose is readily cleaved into monosaccharide for metabolism and subsequent hydrogen generation. Particularly, it can be explained that sucrose supports vigorous growth of the saccharolytic *Clostridium* species. Also, co-culture three *Clostridium* strains can active a cluster of sucrose catabolic genes of *C. beijerinkii*, comprising all genes required for the transport, hydrolysis and subsequent phosphorylation to cleave sucrose to glucose and fructose via a glycosidic linkage. Molecular analysis indicated that *C. beijerinkii* contains four genes: scrARBK, encoding a sucrose-specific transport protein; ScrA, a regulator of the LacI–GalR family (ScrR); the sucrose-6-P hydrolase (ScrB); and a fructokinase (ScrK), respectively. This mechanism was proved in the previous study [43]. Therefore, it could be a reason why the co-culture of the three strains achieved the highest H_2 production (Figure 3C) from sucrose, which was much higher than that by any pure culture or co-culture from glucose (Figure 3A,B).

3.2.2. H_2 production from the food industry wastes

As the unlimited source of natural nutrients, the food industry wastes including cassava stumps (cassava tuber wastes) and distillers wet grains with soluble (DWGS—by-product of ethanol industry) was used as the main substrates to investigate the activity of H_2 production by *C. beijerinckii* ST1, *C. bifermentans* ST4, *C. butyricum* ST5. Figure 4 shows the H_2 yield of the *Clostridium* species in different culture modes from the food industry wastes, including cassava stumps or DWGS. For the cassava stumps, the highest H_2 production of 895.2 ± 5.6 mL/L was given by *C. butyricum* ST5, accounting for 59.2% of the total volume of biogas, and was 1.3-fold that from *C. beijerinckii* NBRC 109359 (658.3 ± 14.4 mL/L) in this study (Figure 4, Table S5, Supplementary Data). Similarly, H_2 production of 805.8 ± 11.5 mL/L and 787.5 ± 9.2 mL/L were obtained from *C. beijerinckii* ST1 and *C. bifermentas* ST4, respectively (Figure 4, Table S5, Supplementary Data). Using the cassava stumps, the H_2 production from co-cultures of two or three *Clostridium* species decreased from 1.7 to 3.6 times compared to that of the pure culture of *C. butyricum* ST5. H_2 production respectively was 248.5 ± 6.8 mL/L, 529.6 ± 8.7 mL/L, and 635.9 ± 7.5 mL/L for the co-cultures of *C. beijerinckii* ST1 and *C. bifermentans* ST4, *C. butyricum* ST5 and *C.beijerinckii* ST4, and *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5 (Figure 4, Table S5, Supplementary Data). This result may be explained by *Clostridium* species competing with each other for resources, which reduce the strains' ability to convert starch to H_2. As an intriguing carbon source, there are many studies on H_2 production from starchy substances. In a previous report [44], H_2 yields of 12.52–9.9 mmol/g starch were obtained by *C. butyricum* CGS2. H_2 yields of 240, 224.4, and 165.2 mL H_2/g of cassava starch were reported for combined cultures of mesophilic organisms taken from three locations in Thailand [45]. Moreover, through the combination of dark- and photo-fermentation, Su et al. obtained H_2 production of 979.9–2541.1 mL/L when using starchy substance concentrations of 10–25 g/L [46].

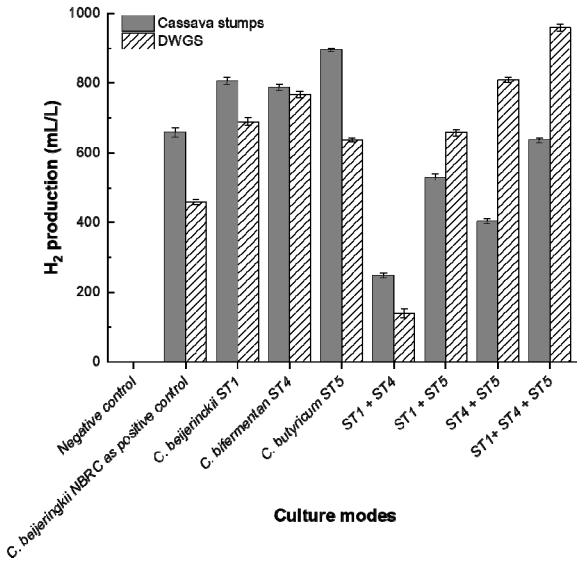

Figure 4. H_2 production from cassava stumps and distillers wet grains with soluble (DWGS) by using pure and co-cultures.

Regarding DWGS as the main substrate (Figure 4 and Table 5S, Supplementary data), a higher H_2 production (765.1 ± 8.6 mL/L) was archived from *C. bifermentans* ST4 compared to that of *C. beijerinckii* ST1 (689.8 ± 11.5 mL/L) or *C. butyricum* ST5 (637.0 ± 8.6 mL/L). In addition, the H_2 production of these strains indicated a higher H_2 production compared with *C. beijerinckii* NBRC 109359 (458.8 ± 7.26 mL/L), that was used as a *Clostridium* strain reference. Interestingly, the highest H_2 production of 809.3 ± 7.9 mL/L was obtained by combining *C. butyricum* ST5 and *C. bifermentans* ST4. In contrast, the co-culture of *C. beijerinckii* ST1 and *C. bifermentans* ST4 gave an H_2 production of 139.5 ± 2.5 mL/L. Perhaps *C. beijerinckii* ST1 and *C. bifermentans* ST4 were better able to cleave organic compounds into acetate, butyric acid, etc. The H_2 production of 658.4 ± 9.4 mL/L obtained with the co-culture of *C. butyricum* ST5 and *C. beijerinckii* ST1 was similar to that from their pure cultures, a result suggesting that these strains are neither inhibited nor interchangeable in hydrogen decomposition. However, in the mix of the three isolated strains *C. beijerinckii* ST1, *C. bifermentans* ST4 and *C. butyricum* ST5, the H_2 production was 959 ± 8.8 mL/L, and the H_2 percentage was 54.5%, which was much higher than that by any pure culture or co-culture of two strains. This result indicated an advantage of combining three trains for H_2 conversion from DWGS as the main substrate.

3.3. Optimization of the key fermentation conditions for H_2 production by mixing three newly isolated Clostridium strains

Temperature is considered one of the most important factors affecting the activities underpinning fermentative H_2 production by H_2-producing bacteria [40,47]. Based on the characterization of different mesophilic and thermophilic H_2-producing bacteria, the optimal temperature for the fermentative H_2 production is expected to vary. For example, the temperature applied to fermentative hydrogen production by different mesophilic H_2-producing bacteria

Clostridium isolates occurs between 30 °C and 40 °C [28,48–50]. The optimum temperature for efficient H_2 production by *C. butyricum* TM-9A was deemed to be 37 °C [51]. For fermentative H_2 production by *C. butyricum* EB6, Chong et al. optimized H_2 production via response surface methodology, and determined the optimal temperature as 36 °C [52]. In addition, the activities of key enzymes related to fermentative H_2 production (such as the hydrogenases) depend considerably upon on temperature. Hence, the optimization of fermentative temperature for the efficient H_2 production is needed.

Nevertheless, substrate concentration is also a critical factor which directly affects the distribution of metabolic products during fermentation [53]. We know that over an appropriate range, increasing the substrate concentration could increase the ability of fermentative H_2 production in H_2-producing bacteria to produce H_2, but much higher substrate concentrations could decrease this activity [54,55]. Furthermore, addressing the effect of substrate concentration on fermentative H_2 production must also be carried out adequately, since this is a critical parameter in determining the economic and technical feasibility of the process [56]. Many studies, therefore, have reported the influence of substrate concentration on fermentative H_2 production [8,57–60]. Understanding the influence of these key factors—temperature and substrate concentration—on bio-H_2 production will help to optimize the operating fermentative H_2 production by a co-culture that mixes the three newly isolated *Clostridium* strains studied here.

In our optimization experiments, the variables X_1 (sucrose concentration), X_2 (fermentation time), and X_3 (fermentation temperature) were taken according to the design in Table S1, and their function was Y_{H2}. The results of these experiments revealed that the highest H_2 yield (1.12 L/L) was obtained under the following specific conditions: a sucrose concentration of 10 g/L, with a fermentation time of 48 h, under a fermentation temperature of 37.5 °C (Table S1). Based on such conditions, it was possible to establish a regression equation describing the relationship between the function Y_{H2} and the variables X_1, X_2, and X_3, as follows:

$$Y_H = 1.10 + 0.072X_1 + 0.066X_2 - 0.10X_3 - 0.010X_1X_2 - 0.030X_1X_3 - 0.057X_2X_3 - 0.14X_1^2 - 0.20X_2^2 - 0.31X_3^2 \quad (2)$$

The ANOVA validated the statistical results and the significance of the fitting model equation for the experimental data (Table 2). The model's *F*-value of 293.95 implied a significant model fit, with the probability of error as noise at <0.0001, which indicated only a 0.01% probability that the model fit the data by chance. To check the significance of each variable, as well as the interaction strength between each independent variable, the *P*-values were used as a tool. As seen in Table 2, the interaction between sucrose concentration (X_1) and fermentation time (X_2) had a low significance ($P > 0.05$), which indicates that these two variables did little to change each other's influence on the response variables. The low *F*-value of 1.07 for the lack of fit implied the latter was not significant relative to the pure error, with 45.07% chance it occurred strictly due to noise. In sum, that lack of fit is insignificant means that the model is robust. The multiple correlation coefficient ($R^2 = 0.9974$), the coefficient of determination (adjusted R^2), and the predicted coefficient (predicted R^2) were also evaluated. The adjusted $R^2 = 0.994$ indicated that 99.4% of the variation in the response variable "Y_{H2}" was explained by the fitted model. These results indicate good agreement between the experimental and predicted values. Hence, this regression model is very reliable as an accurate representation of the experimental data on hydrogen production as reported in this study.

Table 2. Result of ANOVA quadratic model for the H_2 production.

Factors	Statistics				
	Sum of square	Degree of freedom	Mean square	*F*-value	*P*-value
Model	0.91	9	0.10	293.95	< 0.0001
X_1	0.042	1	0.042	122.90	< 0.0001
X_2	0.035	1	0.035	102.63	< 0.0001
X_3	0.086	1	0.086	251.69	< 0.0001
X_1X_2	4.000E-004	1	4.000E-004	1.17	0.3154
X_1X_3	3.600E-003	1	3.600E-003	10.52	0.0142
X_2X_3	0.013	1	0.013	38.65	0.0004
X_1^2	0.083	1	0.083	243.80	< 0.0001
X_2^2	0.17	1	0.17	508.38	< 0.0001
X_3^2	0.40	1	0.40	1169.32	< 0.0001
Residual	2.395E-003	7	3.421E-004		
Lack of fit	1.075E-003	3	3.583E-004	1.09	0.4507
Pure Error	1.320E-003	4	3.300E-004		
Corrected Total	0.91	16			
Coefficient of correlation (R^2): 0.9974					
Coefficient of determination (adjusted R^2): 0.9940					
Coefficient of predicted (predicted R^2): 0.9788					

To investigate the effects of sucrose concentration, temperature, and fermentation time on H_2 production, the three-dimensional response surface and two-dimensional contour plots were constructed to show the relationship between Y_{H2} as a function of its predictor variables (i.e., X_1, X_2, and X_3) (Figure 5). The maximum predicted value is defined by the surface confined in the smallest elliptical contours, which indicates a perfect interaction between the independent variables [61]. Figure 5 shows that the response surface and contour plots indicated that the highest H_2 production (1.13 L/L) was obtained when the sucrose concentration was 11.63 g/L, the fermentation time was 51.13 h, and the fermentation temperature was 36.09 °C.

To confirm the applicability of the above-constructed fermentative model, a confirmation trial was conducted under the optimal conditions at 36.1 °C in medium containing 11.63 g/L sucrose for a culture time of 51.1 h. As a result, the maximum H_2 production was found to be 1.129 L/L. Thus, the difference between the calculated yield according to the model we designed and the empirical yield obtained from a trial of this model was within the allowable range. Hence, this model is deemed useful for further applications.

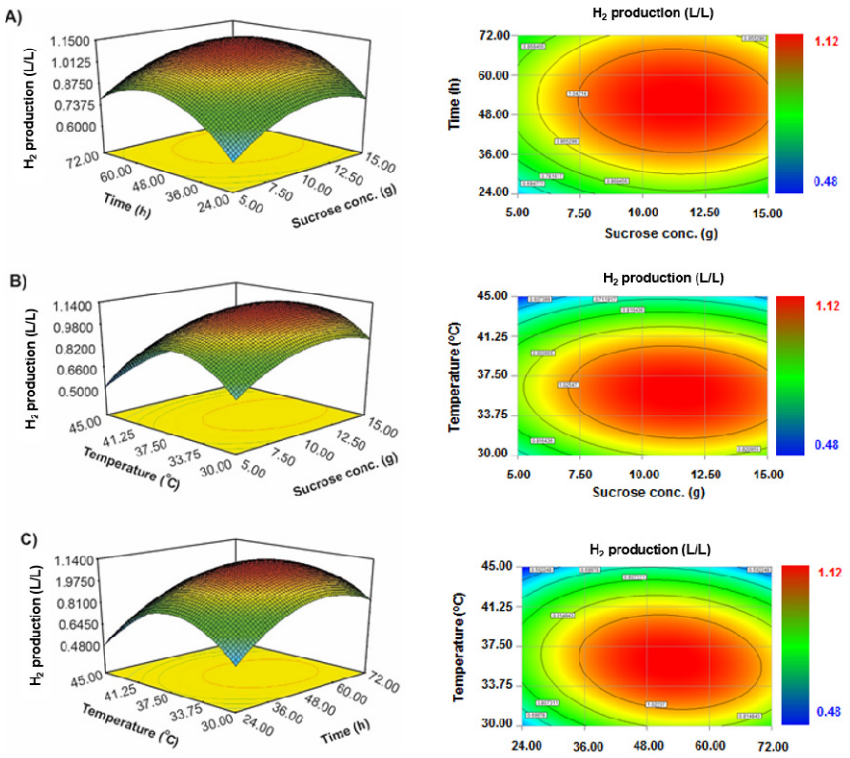

Figure 5. Three-dimensional response surface and two-dimensional contour plots, each of them represents the relationship between H_2 yield and a pair of varied fermentation conditions while the third condition was fixed. A) Effect of time and sucrose concentration on H_2 production. B) Effect of temperature and sucrose concentration on H_2 production. C) Effect of temperature and time concentration on H_2 production.

4. Conclusions

Three novel H_2-producing *Clostridium* strains were isolated and screened from the rumen of cow in Vietnam and identified as *C. beijerinckii* ST1, *C. bifermentans* ST4, and *C. butyricum* ST5. These strains showed a capacity for utilizing a broad range of substrates, including sucrose, glucose, lactose, xylose, and molasses, in addition to food industry wastes such as cassava stumps and distiller wet grains with soluble, for efficient H_2 production in both pure cultures and co-cultures. The highest H_2 production was achieved from a mixed culture consisting of the three strains when compared with the other culture modes (of pure cultures and co-cultures with two strains). Response surface methodology with the Box-Behnken design was used to successfully optimize the operational conditions, including temperature, time, and substrate concentration, for the high-level production of H_2 in this mixed co-culture of the three newly isolated *Clostridium* trains. Statistical analysis revealed that all three variables influenced its H_2 production significantly. A mathematical model was established, with a confidence level of 99%, with $P < 0.0001$. The optimized condition for maximized H_2 production was a sucrose concentration of 11.63 g/L, a fermentation time of 51.13 h, and a fermentation temperature of 36.09 °C that produced 1.13 ± 0.015 L of H_2 per L of medium.

Supplementary material

See supplementary material for Box-Behnken experimental design with 3 independent variables and the corresponding experimental results (Table S1), for H_2 production from 8 isolated strains (Table S2), for physiobiochemical characteristics of *Clostridium* isolated strains (Table S3), for enzymatic activity of isolated *Clostridium* strains (Figure S1), for growth and H_2 production from isolated *Clostridium* strains (Table S4), for a summary of H_2 production by single and co-culture isolated *Clostridium* strains from various substrate (Table S5), and standard curve for H_2 production analysis (Figure S2).

Acknowledgments

The authors thank VNU University of Science for facility support. The Vietnam National University funded this work under grant QG.16.03.

Conflict of interest

The authors declared no potential conflicts of interest respecting to the research, authorship, and pulication of this paper.

References

1. Krishna RH (2013) Review of research on production methods of hydrogen : future fuel. *Eur J Biotechnol Biosci* 1: 84–93.
2. Das D, Veziroglu TN (2008) Advances in biological hydrogen production processes. *Int J Hydrogen Energy* 33: 6046–6057.
3. Gupta SK, Kumari S, Reddy K, et al. (2013) Trends in biohydrogen production: major challenges and state-of-the-art developments. *Environ Technol* 34: 1653–1670.
4. Ntaikou I, Antonopoulou G, Lyberatos G (2010) Biohydrogen production from biomass and wastes via dark fermentation: A review. *Waste Biomass Valori* 1: 21–39 .
5. Rittmann S, Herwig C (2012) A comprehensive and quantitative review of dark fermentative biohydrogen production. *Microb Cell Fact* 11: 115.
6. Peters JW, Schut GJ, Boyd ES, et al. (2015) [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *BBA-Mol Cell Res* 1853: 1350–1369.
7. Hallenbeck PC (2009) Fermentative hydrogen production: Principles, progress, and prognosis. *Int J Hydrogen Energy* 34: 7379–7389.
8. Lee KS, Hsu YF, Lo YC, et al. (2008) Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *Int J Hydrogen Energy* 33: 1565–1572.
9. Masset J, Calusinska M, Hamilton C, et al. (2012) Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium* spp. *Biotechnol Biofuels* 5: 35.
10. Pan CM, Fan YT, Zhao P, et al. (2008) Fermentative hydrogen production by the newly isolated *Clostridium* beijerinckii Fanp3. *Int J Hydrogen Energy* 33: 5383–5391.

11. Wang H, Ma S, Bu H (2014) Fermentative hydrogen production by newly isolated *Clostridium perfringens* ATCC 13124. *J Renew Sustain Energy* 6: 013130.
12. Zhao X, Xing D, Fu N, et al. (2011) Hydrogen production by the newly isolated *Clostridium beijerinckii* RZF-1108. *Bioresour Technol* 102: 8432–8436.
13. Zhang H, Bruns MA, Logan BE (2006) Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. *Water Res* 40: 728–734.
14. Wong YM, Wu TY, Ling TC, et al. (2018) Evaluating new bio-hydrogen producers: *Clostridium perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY. *J Biosci Bioeng* 125: 590–598.
15. Wong YM, Juan JC, Ting A, et al. (2014) High efficiency bio-hydrogen production from glucose revealed in an inoculum of heat-pretreated landfill leachate sludge. *Energy* 72: 628–635.
16. Puniya AK, Singh R, Kamra DN (2015) Rumen Microbiology: From Evolution to Revolution, New Delhi: Springer, 1–379.
17. Krause DO, Denman SE, Mackie RI, et al. (2003) Opportunities to improve fiber degradation in the rumen: Microbiology, ecology, and genomics. *Fems Microbiol Rev* 27: 663–693.
18. Nissilä ME, Tähti HP, Rintala JA, et al. (2011) Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: Effects of different heat treatments. *Int J Hydrogen Energy* 36: 1482–1490.
19. Ratti RP, Botta LS, Sakamoto IK, et al. (2014) Production of H_2 from cellulose by rumen microorganisms: Effects of inocula pre-treatment and enzymatic hydrolysis. *Biotechnol Lett* 36: 537–546.
20. Vos P, Garrity G, Jones D, et al. (2009) Bergey's Manual of Systematic Bacteriology, Vol 3: The Firmicutes, New York: Springer-Verlag.
21. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31: 426–428.
22. Lin PY, Whang LM, Wu YR, et al. (2007) Biological hydrogen production of the genus *Clostridium*: Metabolic study and mathematical model simulation. *Int J Hydrogen Energy* 32: 1728–1735.
23. Chong ML, Sabaratnam V, Shirai Y, et al. (2009) Biohydrogen production from biomass and industrial wastes by dark fermentation. *Int J Hydrogen Energy* 34: 3277–3287.
24. Karube I, Urano N, Matsunaga T, et al. (1982) Hydrogen production from glucose by imoobilized growing cells of *Clostridium butyricum*. *Eur J Appl Microbiol Biotechnol* 16: 5–9.
25. Karube I, Matsunaga T, Tsuru S, et al. (1976) Continuous hydrogen production by immobilized whole cells of *Clostridium Butyricum*. *BBA-Gen Subj* 444: 338–343.
26. Beckers L, Hiligsmann S, Hamilton C, et al. (2010) Fermentative hydrogen production by *Clostridium butyricum* CWBI1009 and *Citrobacter freundii* CWBI952 in pure and mixed cultures. *Biotechnol Agron Soc Environ* 14: 541–548.
27. Beckers L, Hiligsmann S, Lambert SD, et al. (2013) Improving effect of metal and oxide nanoparticles encapsulated in porous silica on fermentative biohydrogen production by *Clostridium butyricum*. *Bioresour Technol* 133: 109–117.
28. Beckers L, Masset J, Hamilton C, et al. (2015) Investigation of the links between mass transfer conditions, dissolved hydrogen concentration and biohydrogen production by the pure strain *Clostridium butyricum* CWBI1009. *Biochem Eng J* 98: 18–28.

29. Calusinska M, Hamilton C, Monsieurs P, et al. (2015) Genome-wide transcriptional analysis suggests hydrogenase- and nitrogenase-mediated hydrogen production in *Clostridium butyricum* CWBI 1009. *Biotechnol Biofuels* 8: 27.
30. Hiligsmann S, Beckers L, Masset J, et al. (2014) Improvement of fermentative biohydrogen production by *Clostridium butyricum* CWBI1009 in sequenced-batch, horizontal fixed bed and biodisc-like anaerobic reactors with biomass retention. *Int J Hydrogen Energy* 39: 6899–6911.
31. Masset J, Hiligsmann S, Hamilton C, et al. (2010) Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently isolated strain of hydrogen-producing *Clostridium butyricum* CWBI1009. *Int J Hydrogen Energy* 35: 3371–3378.
32. Jenol MA, Ibrahim MF, Yee PL, et al. (2014) Sago biomass as a sustainable source for biohydrogen production by *Clostridium butyricum* A1. *Bioresources* 9: 1007–1026.
33. Seelert T, Ghosh D, Yargeau V (2015) Improving biohydrogen production using *Clostridium beijerinckii* immobilized with magnetite nanoparticles. *Appl Microbiol Biotechnol* 99: 4107–4116.
34. Wang X, Hoefel D, Saint CP, et al. (2007) The isolation and microbial community analysis of hydrogen producing bacteria from activated sludge. *J Appl Microbiol* 103: 1415–1423.
35. Koskinen PEP, Beck SR, Örlygsson J, et al. (2008) Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas. *Biotechnol Bioeng* 101: 679–690.
36. Mitchell RJ, Kim JS, Jeon BS, et al. (2009) Continuous hydrogen and butyric acid fermentation by immobilized *Clostridium tyrobutyricum* ATCC 25755: Effects of the glucose concentration and hydraulic retention time. *Bioresour Technol* 100: 5352–5355.
37. Chen WM, Tseng ZJ, Lee KS, et al. (2005) Fermentative hydrogen production with CGS5 isolated from anaerobic sewage sludge. *Int J Hydrogen Energy* 30: 1063–1070.
38. Takara K, Ushijima K, Wada K, et al. (2007) Phenolic compounds from sugarcane molasses possessing antibacterial activity against cariogenic bacteria. *J Oleo Sci* 56: 611–614.
39. Ding J, Liu BF, Ren NQ, et al. (2009) Hydrogen production from glucose by co-culture of *Clostridium Butyricum* and immobilized *Rhodopseudomonas faecalis* RLD-53. *Int J Hydrogen Energy* 34: 3647–3652.
40. Geng A, He Y, Qian C, et al. (2010) Effect of key factors on hydrogen production from cellulose in a co-culture of *Clostridium thermocellum* and *Clostridium thermopalmarium*. *Bioresour Technol* 101: 4029–4033.
41. Liu Y, Yu P, Song X, et al. (2008) Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. *Int J Hydrogen Energy* 33: 2927–2933.
42. Chang JJ, Chou CH, Ho CY, et al. (2008) Syntrophic co-culture of aerobic *Bacillus* and anaerobic *Clostridium* for bio-fuels and bio-hydrogen production. *Int J Hydrogen Energy* 33: 5137–5146.
43. Reid SJ, Abratt VR (2005) Sucrose utilisation in bacteria: Genetic organisation and regulation. *Appl Microbiol Biotechnol* 67: 312–321.
44. Chen SD, Lee KS, Lo YC, et al. (2008) Batch and continuous biohydrogen production from starch hydrolysate by *Clostridium* species. *Int J Hydrogen Energy* 33: 1803–1812.
45. O-Thong S, Hniman A, Prasertsan P, et al. (2011) Biohydrogen production from cassava starch processing wastewater by thermophilic mixed cultures. *Int J Hydrogen Energy* 36: 3409–3416.

46. Su H, Cheng J, Zhou J, et al. (2009) Improving hydrogen production from cassava starch by combination of dark and photo fermentation. *Int J Hydrogen Energy* 34: 1780–1786.
47. Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy* 34: 799–811.
48. Wang X, Monis PT, Saint CP, et al. (2009) Biochemical kinetics of fermentative hydrogen production by *Clostridium butyricum* W5. *Int J Hydrogen Energy* 34: 791–798.
49. An D, Li Q, Wang X, et al. (2014) Characterization on hydrogen production performance of a newly isolated *Clostridium beijerinckii* YA001 using xylose. *Int J Hydrogen Energy* 39: 19928–19936.
50. Yin Y, Wang J (2017) Isolation and characterization of a novel strain *Clostridium butyricum* INET1 for fermentative hydrogen production. *Int J Hydrogen Energy* 42: 12173–12180.
51. Junghare M, Subudhi S, Lal B (2012) Improvement of hydrogen production under decreased partial pressure by newly isolated alkaline tolerant anaerobe, *Clostridium butyricum* TM-9A: Optimization of process parameters. *Int J Hydrogen Energy* 37: 3160–3168.
52. Chong ML, Rahim RA, Shirai Y, et al. (2009) Biohydrogen production by *Clostridium butyricum* EB6 from palm oil mill effluent. *Int J Hydrogen Energy* 34: 764–771.
53. Yu HQ, Fang HH (2001) Acidification of mid- and high-strength dairy wastewaters. *Water Res* 35: 3697–3705.
54. Lo YC, Chen WM, Hung CH, et al. (2008) Dark H_2 fermentation from sucrose and xylose using H_2-producing indigenous bacteria: Feasibility and kinetic studies. *Water Res* 42: 827–842.
55. Ginkel SV, Sung S, Lay JJ (2001) Biohydrogen production as a function of pH and substrate concentration. *Environ Sci Technol* 35: 4726–4730.
56. Hawkes FR, Dinsdale R, Hawkes DL, et al. (2002) Sustainable fermentative hydrogen production: Challenges for process optimisation. *Int J Hydrogen Energy* 27: 1339–1347.
57. Lay JJ (2001) Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. *Biotechnol Bioeng* 74: 280–287.
58. Lin CY, Chang CC, Hung CH (2008) Fermentative hydrogen production from starch using natural mixed cultures. *Int J Hydrogen Energy* 33: 2445–2453.
59. Chen WH, Chen SY, Khanal SK, et al. (2006) Kinetic study of biological hydrogen production by anaerobic fermentation. *Int J Hydrogen Energy* 31: 2170–2178.
60. Park JH, Kim DH, Kim SH, et al. (2018) Effect of substrate concentration on the competition between *Clostridium* and *Lactobacillus* during biohydrogen production. *Int J Hydrogen Energy* 43: 11460–11469.
61. Guo WQ, Ren NQ, Wang XJ, et al. (2009) Optimization of culture conditions for hydrogen production by *Ethanoligenens harbinense* B49 using response surface methodology. *Bioresour Technol* 100: 1192–1196.