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Original Article

Isolation and Characterization of a New High-Yield Hydrogen-Producing Strain, *Clostridium sulfidigenes* CS3, from Anaerobic Sludge in the To Lich River

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Abstract: Hydrogen is widely recognized as a renewable and clean energy source, with its critical role as a feedstock in various industries, leading to a significant rise in hydrogen production demand in recent years. In this context, a high-yield hydrogen-producing mesophilic bacterium, strain CS3, was isolated from sludge and identified as *Clostridium sulfidigenes* CS3 through 16S rRNA gene analysis and physio-biochemical testing, revealing novel characteristics of this species. This study systematically investigated various factors influencing biological hydrogen production through fermentation, including medium components (carbon and nitrogen sources) and environmental conditions (initial pH, incubation temperature, duration, and agitation). The study also explored methods to boost hydrogen yield. The maximum yield of 2.1 mol H_2/mol glucose was obtained under optimal conditions: A medium with 10 g/L glucose, an initial pH of 7.0, incubation at 37 °C for 48 hours, and agitation at 200 rpm, with supplementation of organic nitrogen sources (10 g/L peptone and 10 g/L yeast extract). The performance of *C. sulfidigenes* CS3 was compared with other known hydrogen-producing strains, highlighting the superior hydrogen productivity of this species. This is also considered a discovery in the hydrogen-producing ability of this species. The findings suggest that *C. sulfidigenes* CS3 could be a promising candidate for biohydrogen production, providing an environmentally friendly alternative to fossil fuels.

Keywords: Biohydrogen, *Clostridium sulfidigenes*, dark fermentation, sludge.

1. Introduction

Today, most global energy demands are fulfilled by fossil fuels, which are rapidly being depleted. Moreover, the greenhouse gas emissions from fossil fuels, along with other environmental issues such as global warming, climate change, and ozone layer depletion, have created an urgent need for renewable energy sources [1]. Hydrogen emerges as a promising alternative to address these challenges. It avoids

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contributing to the greenhouse effect, producing only heat and water upon combustion, and offers a high energy yield of 286 kJ/mol-at least twice that of any hydrocarbon fuel [2]. As an environmentally friendly energy resource, hydrogen represents an ideal alternative to fossil fuels, with the highest energy content per unit weight among all naturally occurring fuels [3].

Biological hydrogen (H_2) production can be classified according to the metabolic pathways used by various microorganisms. One of the key methods for biohydrogen generation is dark fermentation [4]. This is a catabolic process in which bacteria decompose sugars and proteins into carboxylic acids, hydrogen gas, carbon dioxide, and organic solvents. Importantly, this process takes place exclusively in anaerobic environments [5]. Dark-fermentative hydrogen-producing bacteria, including *Clostridium, Ethanoligenens*, *Enterobacter*, *Citrobacter*, and even some aerobes like *Alcaligenes* and *Bacillus*, have been isolated from bioreactors and natural environments. Among these, *Clostridium* spp. and *Enterobacter* spp. are the most extensively studied. *Clostridium* species are Gram-positive, rod-shaped, strict anaerobes that form endospores, while *Enterobacter* species are Gram-negative, rod-shaped, and facultatively anaerobic. *Clostridium* stands out as one of the most effective hydrogen producers within the *Firmicutes* phylum, with many strains having been isolated and researched, highlighting its significant potential for renewable energy applications [6]. This report focuses on the isolation and characterization of *C. sulfidigenes* CS3, which was isolated from the sludge of the To Lich River. The study explores the fermentative hydrogen production capabilities of this isolate under various culture conditions to identify suitable conditions for fermentative hydrogen production.

2. Materials and Methods

2.1. Location and Pre-treatment

The bacterium used in this study was isolated from the sludge of the To Lich River located in Hanoi, Vietnam. Using a sterile syringe, the mud sample is taken from the deepest layer, at least 5-10 cm from the surface, to ensure the least oxygen environment. Then, the syringe head is sealed with wax paper and immediately placed in a sealed container with a lid and stored at 4 ^oC. Firstly, sludge was diluted at concentrations of 10^{-1} to 10^{-3} with sterilized distilled water, filtering through filter paper to remove other impurities to collect only the solution containing bacteria, and pre-treating it with a sample heater at 100 ^oC for 30 minutes to inactivate hydrogen consumers and harvest spore-forming anaerobic bacteria.

2.2. Bacterial Strain Isolation

The basic medium used for enrichments, isolation, and cultivation of hydrogen-producing strains were routinely grown in Peptone Yeast Extract (PY) agar medium (1 L) contained: Peptone 10 g; yeast extract 10 g; glucose 10 g; resazurin 1 mg; salt solution 40 ml (composition in g/100 ml: $K_2HPO_4.12H_20$-0.1, KH_2PO_4 -0.1, $NaHCO_3$-1.0, NaCl-0.2, $CaCl_2$. 2 H_2O-0.02, $MgSO_4$. $7H_2O$-0.02), pH 6.5 [7]. This medium was autoclaved at 121 ^oC for 20 minutes; the medium consisted of a basal medium that was flushed with 99.99% N_2 gas to remove oxygen [8]. The samples were enriched in PY medium, and shaken at 35 ^oC, for 48 hours, shaking speed of 100 rpm. Each 6 mL of medium was put into a 12 ml sterilized serum bottle, then closed with a rubber cap and an aluminum cap on the outside.

After the 3rd enrichment, the shake culture is spread on a plate on PY agar medium incubation for 48 hours, by the following Whitley A55 anaerobic Workstation (Don Whitley Scientific, United Kingdom).

Pure cultures for biohydrogen production

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 a 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.

2.3. Identify Bacteria Based on Bergey's Identification Keys

From the cultivated Petri dish in anaerobic condition in the Whitley A55 anaerobic Workstation (Don Whitley Scientific, United Kingdom), one type of separated colony was chosen based on the difference from each other by shape, size, color, surface, edge, and elevation.

Physio-biochemical characteristics

Pure isolated strains were tested for their morphology, physiology, and biochemistry properties following the standard protocol that has been conventionally used in bacterial systematic [9].

The cell morphology of the selected strain was observed under a scanning electron microscope (SEM, JEOL, JSM-5410LV in 69 Institute, Ministry of National Defence).

2.4. 16S rRNA Nucleotide Sequencing

Genomic DNA extraction and purification were performed using Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions, and the purified DNA was then used as the template for PCR. Each PCR reaction mixture consisted of approximately 4 µL of DNA, 3 µL of 10X buffer, 3 µL each of dCTP, dGTP, dATP, and dTTP, 1.5 µL (10 pmol) of forward and reverse primers, 0.7 µL of Taq DNA polymerase, and 16.3 µL of H_2O, making up a final volume of 30 µl. The purified genomic DNA was tested by electrophoresis on a 1% agarose gel, stained with EtBr, and visualized under UV light.

The 16S rRNA gene was amplified by PCR using a bacterial domain-specific universal set of primers: 27F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 1527R (5′- 5'-AAAGGAGGTGATCCAGCC-3′). The PCR program included an initial denaturation step at 94 °C for 5 minutes, followed by 30 cycles of 30 seconds at 94 °C, 45 seconds at 62 °C, and 90 seconds at 72 °C, with a final extension step at 72 °C for 5 minutes. The PCR products were then sent for sequencing by First BASE. The resulting sequences were analyzed and compared using BLAST software against the NCBI database (http://blast.ncbi.nlm.gov/Blast.cgi) to identify the strain with the closest sequence match of the type strain. The 16S rDNA sequence of these strains was analyzed with BioEdit v7.2 software and aligned against existing sequences in the GenBank database by using the BLAST program. A phylogenetic tree was constructed in IQ-TREE 2.2.0 and bootstrapped with 1000 replicates. A visualization of the phylogenetic tree was generated using the iTOL v6 online tool.

2.5. Analytical Methods and Calculations

Growth was monitored by the optical density (OD_{600}) of the sterile medium as the control. Hydrogen gas in the headspace was sampled with a gas-tight syringe and determined by gas chromatography (GC) using the method described by Li et al., [10]. The total biogas production was recorded daily, using the water displacement method [11].

The glucose concentration was determined with the dinitrosalicylic acid (DNS) colorimetric method as described by Miller [12].

All analyses were run in triplicate.

3. Results and Discussions

3.1. Isolation and Identification of a Hydrogen-Producing Isolate

From 2 mud samples collected at To Lich River, 3 *Clostridium* sp. strains were isolated and purified (Table 1).

These 3 strains were tested for hydrogen synthesis ability, the results are shown in Table 1. Of which, strain CS3 had the highest hydrogen formation ability and was selected for further studies. This train was tested for morphological, physiological, and metabolic characteristics [9]. Even though this method is not exact, but is the first step in the study of microbial classification.

Table 1. Bacterial strains isolated from samples

Bacterial strains	Sampling location	H_2 yield (mol H_2/ mol glucose)	OD_{600}
CS 1	To Lich (Cau Giay)	1.15	0.898
CS 2	To Lich (Thanh Xuan)	0.84	0.723
CS 3	To Lich (Thanh Xuan)	**1.49**	1.318

The identification of bacteria using Bergey's Manual of Determinative Bacteriology.

After being isolated, the morphological, physiological, and metabolic characteristics of strains were primarily estimated following Bergey's Identification keys. The result is shown in Figure 1

Figure 1. Gram staining - Microscopic view of the bacterium (left) (x40) and Scanning electron microscopy (SEM) image (right) of strain CS3 under a JEOL, JSM-5410LV electron microscope instrument operating at 15kV and 7,500X.

Table 2. Comparison between the characteristics of strain CS3 and its closest phylogenetic relatives

Strains CS3 (In this study)	Source	Gram stain	String Test	Mobility	Catalase Test	Growth temperature	pH
	River sediment	+	+	+	-	35 °C	6.5
	Metabolite prodution (H_2S)	Starch hydrolyzed	Thiosulfate reduction	Indol produce	Oxidase Test	MR Test	Endospore
	ND	+	ND	+	-	+	+
SGB2 [13]	Source	Gram stain	String Test	Mobility	Catalase Test	Growth temperature	pH
	Pond sediment	+	+	+	-	34 °C	6.6
	Metabolite prodution (H_2S)	Starch hydrolyzed	Thiosulfate reduction	Indol produce	Oxidase Test	MR Test	Endospore
	+	+	+	ND	-	ND	+

This analysis highlights that while both strains share several physiological traits (e.g., motility, endospore formation, oxidase absence, growth mesophile bacteria), but they also have notable biochemical differences, especially in metabolite production and enzymatic activities, which could affect their ecological roles or applications in bioprocesses.

The identification of bacteria using 16S rRNA gene sequences.

BLAST search on the GenBank revealed that the full length of the 16S rRNA sequence of strain CS3 has 99.8% similarity to that of *Clostridium sulfidigenes* (SGB2) (Figure 2). Based on the biochemical and physiological characteristics, there are similarities and differences as mentioned above, and especially based on the 16S rRNA gene sequencing, strain CS3 is close to strain *C. sulfidigenes* SGB2 with a similarity level of 99.8%. So this strain CS3 was named *C. sulfidigenes* CS3 with accession number SUB14851242 in GenBank (Figure 2).

C. sulfidigenes, a mesophilic, proteolytic, thiosulfate- and sulfur-reducing bacterium, can produce hydrogen due to several metabolic characteristics inherent to the *Clostridium* genus and its specific biochemical pathways. Some of the key reasons why *C. sulfidigenes* can produce hydrogen as follows:

i) Like other *Clostridia* species, it is an anaerobic, fermentative that metabolizes organic substrates (such as sugars or proteins) to generate energy. During this process, organic compounds are broken down into simpler products like short-chain fatty acids, alcohols, CO_2, and H_2.;

ii) Hydrogen production is a key part of this fermentation process, particularly during the breakdown of pyruvate to acetyl-CoA.

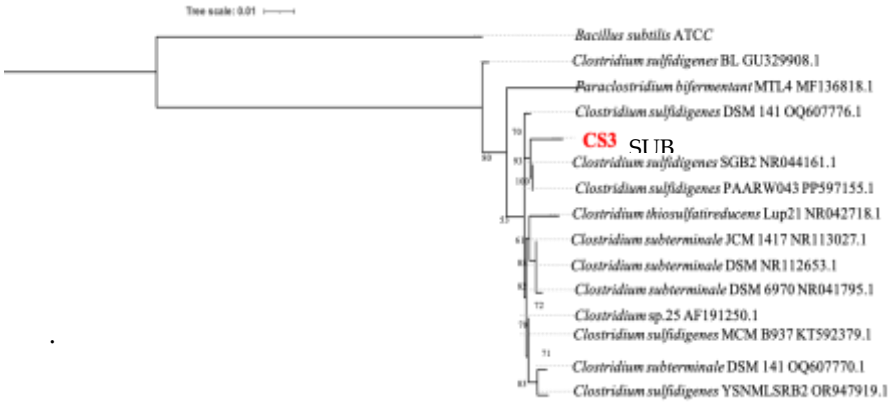

Figure 2. The phylogenetic tree shows the relationship between strain CS3 and related species based on 16S rDNA sequence analysis.

The bacteria use enzymes such as hydrogenase to catalyze the production of hydrogen gas (H_2) from protons (H^+) and electrons. In some cases, hydrogen is produced as an intermediate during the reduction of sulfur-containing compounds, particularly thiosulfate ($S_2O_3^{2-}$). That's why *C. sulfidigenes* produces hydrogen through its fermentative metabolism, aided by hydrogenase enzymes, while balancing electron flow during the reduction of sulfur and thiosulfate compounds. These processes allow the bacterium to maintain energy production and redox balance under anaerobic conditions.

3.2. Effect of Different Substrates and Glucose Concentration on Hydrogen Production by C. sulfidigenes CS3

Carbohydrates serve as essential carbon sources for fermentative hydrogen production. Efficient utilization of various carbohydrates is key to optimizing bio-hydrogen yields from different substrates. Figure 3-A demonstrates that certain strains can metabolize a range of sugars, including saccharose, maltose, glucose, cellulose, xylose, and lactose, to support growth. A positive correlation between H_2 yield and OD_{600} is observed, indicating that higher

biomass growth typically aligns with greater hydrogen production. Simpler substrates, like glucose and saccharose, result in higher H_2 yields and enhanced microbial growth compared to more complex substrates such as cellulose and maltose. Glucose stands out as the most effective substrate for both hydrogen production and microbial proliferation (Figure 3-A). In contrast, more complex substrates, like cellulose and maltose, are less efficient due to the additional metabolic processes required to break them down. The complexity and structure of the substrate significantly impact the efficiency of hydrogen production by CS3 strain.

Besides, nitrogen also is a vital nutrient for growth, and *C. sulfidigenes* CS3 can utilize various organic and inorganic nitrogen sources, including beef extract, peptone, yeast extract, NH_4NO_3, and NH_4Cl. Among the five nitrogen sources tested, the medium supplemented with yeast extract and peptone produced the highest hydrogen yield of 1.57 mol H_2/mol glucose and resulted in the lowest final pH of 4.81 (Figure 3-B). Meanwhile, hydrogen production using nitrogen from inorganic sources such as NH_4Cl and NH_4NO_3 performed poorly, with little to no mol H_2/mol glucose recorded.

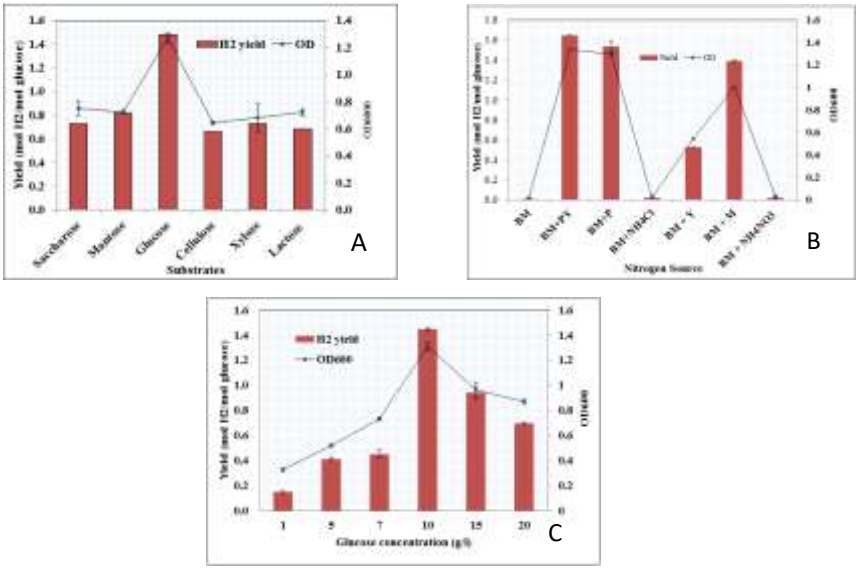

Figure 3. Effect of different substrates (A- carbon sources; B- nitrogen sources) and C-glucose concentration on hydrogen production by CS3 strain.

Our experimental findings suggest that an appropriate nitrogen source can enhance both cell growth and hydrogen production, with yeast extract being particularly effective as an organic nitrogen source for strain CS3. The lower hydrogen yield observed with a single nitrogen source implies that combining nitrogen sources may be more beneficial for cell growth and hydrogen production. Further research is needed to explore the effects of different nitrogen source combinations on these processes.

Organic loading plays an important role in the fermentative hydrogen-producing process. It affects both H_2-producing yield as well as cell growth. The initial glucose concentrations affected H_2 production and growth of strain CS3 (Figure 3-C). The hydrogen yield and production rate increased with increasing glucose supplementation from 1 g/L to 20 g/L, and reached a maximum of 1.57 mole H_2/mol glucose at 10 g/L of glucose. When the substrate concentration increased from 10 to 20 g/L, the

cumulative volumes of H_2 per liter increased gradually, but the H_2 yield per mole of carbon source decreased. *C. sulfidigenes* CS3 and other strains are using the same amount of 10 g/L glucose such as *C. beijerinkii* str. RZF-1108. Compared to some strains such as *Clostridium* sp.str.6A-5 using 16 g/L, *C. butyricum* EB6 using 15.7 g/L, 10 g/L of *C. sulfidigenes* CS3 strain is possible to save the cost for fermentative hydrogen production [14].

3.3. Effect of some Culture Conditions on Hydrogen Production

Temperature is a critical ecological factor that significantly impacts the physiological activities of microorganisms, including the production of metabolic products. In our study on the adaptation of *C. sulfidigenes* CS3 to different temperature ranges. Figure 4 -A illustrates the relationship between temperature and hydrogen production yield. The data indicate that temperature has a strong influence on hydrogen production. The yield increased steadily from 25 °C to 37 °C, with hydrogen production from 0.3 to 1.63 mol H_2/mol glucose, reaching its peak at 37 °C. This figure demonstrates a clear temperature dependency for both growth (OD_{600}) and hydrogen production, with an optimal range between 35 °C and 37 °C. Beyond this range, particularly at higher temperatures, both processes are severely inhibited, suggesting that temperature control is critical for maximizing both microbial growth and hydrogen production in this system.

Figure 4-B represents the relationship between pH and hydrogen yield (mol H_2/mol glucose) along with cell density (OD_{600}) for a particular microorganism. The hydrogen yield is highest at pH 7, which suggests that neutral conditions are most conducive for efficient hydrogen production.

Similarly, growth (OD_{600}) peaks at pH 7, implying that this pH is also ideal for cellular proliferation. Under acidic conditions (pH 4.5 to 5), both growth and hydrogen production are significantly inhibited, indicating that this microorganism is sensitive to acidic environments. In contrast, under alkaline conditions (pH 7.5 to 8), both hydrogen production and growth decline sharply, suggesting that the microorganism is also inhibited by higher pH levels. pH 7 is the optimal condition for both growth and hydrogen production for this microorganism. Deviations from this neutral pH, whether more acidic or alkaline, lead to a marked decrease in both hydrogen yield and cell density. Maintaining a neutral pH is thus crucial for maximizing the microorganism's hydrogen production and growth in this system (Figure 4-B).

Figure 4-C shows the relationship between time (h) and hydrogen yield (mol H_2/mol glucose) as well as cell density (OD_{600}) over 72 hours. The optimal time for hydrogen production is 48 hours when both hydrogen yield and cell density (OD_{600}) reach their maximum. This period likely represents the most efficient metabolic phase for the microorganism. After 48 hours, both hydrogen yield and OD_{600} decline, indicating that the system is moving towards the stationary or death phase, likely due to nutrient limitations or inhibitory effects of metabolic by-products.

The lag phase occurs during the first 6-12 hours, where both hydrogen production and growth are minimal. The sharp decline in hydrogen production after 48 hours suggests that prolonged cultivation may not be beneficial for sustained hydrogen yield, as the efficiency decreases with time. The optimal hydrogen production and growth for this microorganism occur at around 48 hours. After this point, both hydrogen yield and growth decline, likely due to resource depletion or inhibitory by-products. For maximum efficiency in hydrogen production, the fermentation process should likely be harvested or refreshed around the 48-hour mark.

Overall, the agitation rate significantly impacted both cell growth and hydrogen production. In an anaerobic strain, the increased shaking speed enhances nutrient absorption, but excessively high aeration rates can inhibit biomass growth [15].

Cultivation of the microorganism was conducted at pH 7.0, 37 °C, with a glucose concentration of 10 g/L, and for 48 hours under various agitation speeds (Figure. 4-D). Hydrogen production yield varied with changes in shaking speed, with the maximum yield achieved at 200 rpm. Both higher and lower speeds were less effective for hydrogen production. The results of this study are small different from the study of Dwierra et al., (2000) which showed that the optimal conditions for hydrogen biosynthesis of *C. paraputrificum* M-21 strain were pH 6.5, 45 ºC, 250 rpm stirring speed in a culture volume of 500 mL achieving 1.9 mol hydrogen/ mol GLcNAc [14].

According to the above results, growth is directly proportional to hydrogen production efficiency. However, this is not always true. In some cases, hydrogen produced can inhibit the growth of the producing strain. In many *Clostridium*, H_2 accumulation has an inhibitory effect on growth due to thermodynamic and metabolic constraints. As H_2 builds up, it raises the partial pressure (pH_2), shifting the equilibrium and making further H_2 production unfavorable. Understanding and managing H_2 accumulation is crucial not only for optimizing growth but also for enhancing biohydrogen production in industrial applications [16].

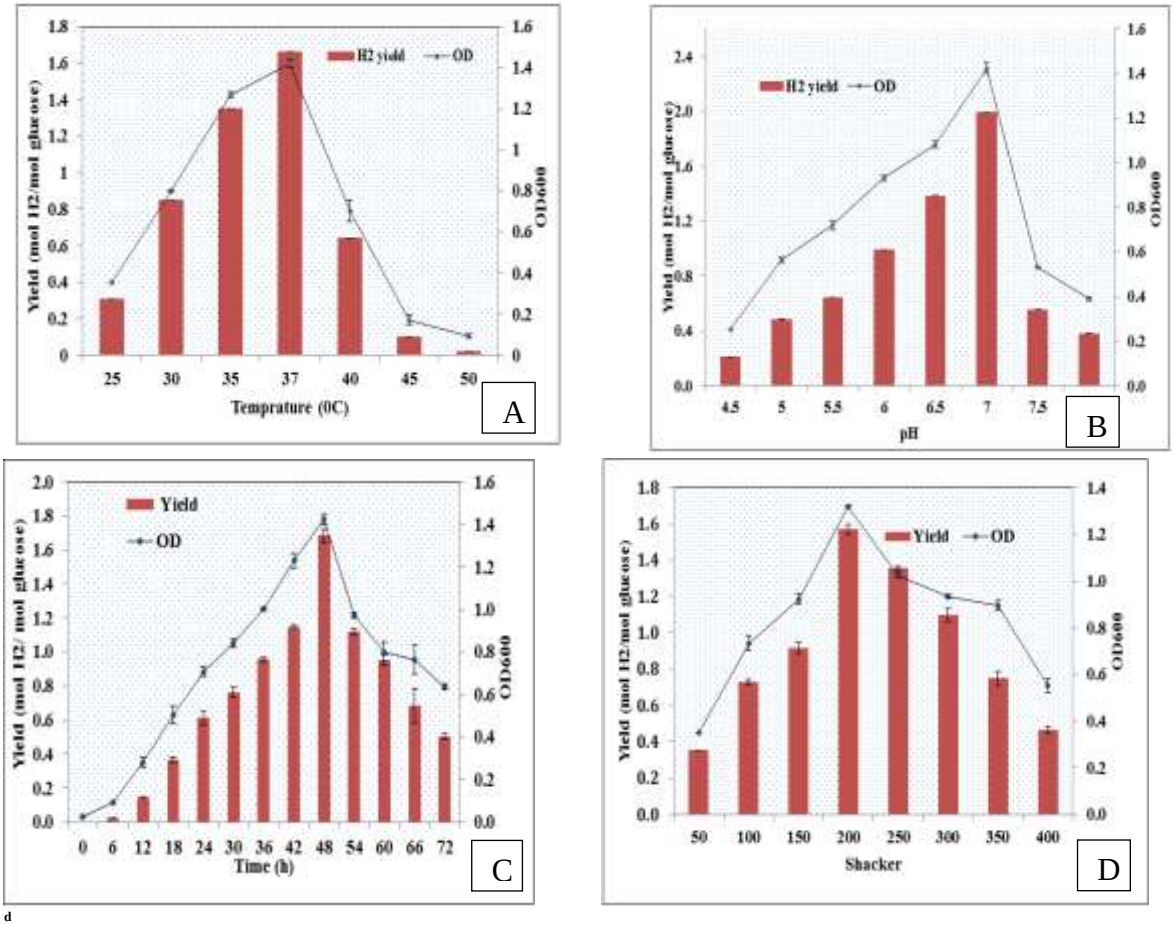

Figure 4. Effect of culturing conditions on the growth and H_2 production by strain CS3.

A- Effect of culture temperature; B- Effect of culture temperature;
C- Effect of time; D- Effect of agitation speed on H_2 production.

3.4. Hydrogen Production under Optimized Conditions

Under the following optimized conditions:

Factor	Optimized condition	Yield (mol H_2/ mol glucose)
Substrate	Glucose	2.1
Concentration substrate	10 g/l	
pH	7	
Temperature	37 °C	
Nitrogen	Peptone + yeast extract	
Cultivation Time	48h	
Shakes	200 rpm	

The maximum yield of hydrogen was 2.1 mol H_2/ mol glucose. The hydrogen yield found in this study is also higher than the 0.1 mol H_2/mol glucose reported for the pure culture of *C. bifermentans* of Wang et al., [15]. Our research identifies optimized conditions for hydrogen production using *C. sulfidigenes* CS3, resulting in a 5% increase in hydrogen performance compared to results reported by Wang et al., [17]. These findings highlight the potential of this bacterium for efficient hydrogen production. As published studies have shown glucose is the most widely used carbohydrate source, with hydrogen yields ranging from 0.1 mol/mol glucose to 3.9 mol/mol glucose. The highest hydrogen yield was obtained from *C. perfringens* ATCC 13124 strains [17], achieving 97.5% of the theoretical hydrogen yield from glucose. Because glucose is a monosaccharide that is directly utilized by bacteria with fewer metabolic steps. This result was also confirmed in the study of the same group of authors published in 2018, showing that *C. bifermetans* ST4 strain was capable of synthesizing 2.74 mol H_2/ mol glucose [18]. In contrast, fructose is also a monosaccharide, but the hydrogen yield obtained (fructose 0.86 to 1.2 mol/mol) was significantly lower than that of glucose [19].

4. Conclusion

In summary, a hydrogen-producing strain, identified as *C. sulfidigenes* CS3, has been isolated. This strain shows great potential for hydrogen production via fermentation, with a maximum yield of 2.1 mol H_2/mol glucose. The optimal conditions for hydrogen production from a synthetic medium using *C. sulfidigenes* CS3 were found to be pH 7.0, 10 g/L glucose, 37 °C, an organic nitrogen source (peptone and yeast extract), and a shaking rate of 200 rpm. These findings indicate that *C. sulfidigenes* CS3 is a promising candidate for hydrogen production.

References

[1] R. Wang, W. Zong, C. Qian, Y. Wei, R. Yu, Z. Zhou, Isolation of *Clostridium perfringens* Strain W11 and Optimization of its Biohydrogen Production by Genetic Modification, International Journal of Hydrogen Energy, Vol. 36, No. 19, 2011, pp. 12159-12167, https://doi.org/10.1016/j.ijhydene.2011.06.105.

[2] M. Cai, J. Liu, Y. Wei, Enhanced Biohydrogen Production from Sewage Sludge with Alkaline Pretreatment, Environmental Science & Technology, Vol. 38, No. 11, 2004, pp. 195-202, https://doi.org/10.1021/es0349204.

[3] N. Ren, A. Wang, G. Cao, J. Xu, L. Gao, Bioconversion of Lignocellulosic Biomass to Hydrogen: Potential and Challenges, Biotechnology Advances, Vol. 27, No. 6, 2009, pp. 1051-1060, https://doi.org/10.1016/j. biotechadv.2009.05.007.

[4] P. C. Hallenbeck, J. R. Benemann, Biological Hydrogen Production, Fundamentals and Limiting Processes, International Journal of Hydrogen Energy, Vol. 27, No. 11+12, 2002, pp. 1185-1193, https://doi.org/10.1016/S0360-3199(02)00131-3.

[5] D. B. Levin, L. Pitt, M. Love, Biohydrogen Production: Prospects and Limitations to Practical Application, International Journal of Hydrogen Energy, Vol. 29, No. 2, 2004, pp. 173-185, https://doi.org/10.1016/S0360-3199(03)00094-6.

[6] C. Y. Lin, S. H. Shei, Heavy Metal Effects on Fermentative Hydrogen Production Using Natural Mixed Microflora, International Journal of Hydrogen Energy, Vol. 33, No. 2, 2008, pp. 587-593, https://doi.org/10.1016/j.ijhydene.2007.09. 030.

[7] H. H. P. Fang, C. Li, T. Zhang, Acidophilic Biohydrogen Production from Rice Slurry, International Journal of Hydrogen Energy, Vol. 31, No. 6, 2006, pp. 683-692, https://doi.org/ 10.1016/j.ijhydene.2005.07.005.

[8] L. B. Kamalaskar, P. K. Dhakephalkar, K. K. Meher, D. R. Ranade, High Biohydrogen Yielding *Clostridium* sp. DMHC-10 Isolated from Sludge of Distillery Waste Treatment Plant, International Journal of Hydrogen Energy, Vol. 35, No. 19, 2010, pp. 10639-10644, https://doi.org/10.1016/ j.ijhydene.2010.05.020.

[9] P. Vos, G. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. H. Schleifer, W. B. Whitman, Bergey's Manual of Systematic Bacteriology, Volume 3: The Firmicutes, Springer-Verlag New York, 2009, https://doi.org/ 10.1007/978-0-387-68489-5.

[10] S. Li, C. Lai, Y. Cai, X. Yang, S. Yang, M. Zhu, J. Wang, X. Wang, High Efficiency Hydrogen Production from Glucose/Xylose by the *ldh*-Deleted *Thermoanaerobacterium* Strain, Bioresource Technology, Vol. 101, No. 22, 2010, pp. 8718-8724, https://doi.org/10.1016/j.biortech. 2010.06.111.

[11] G. Lettinga, J. Field, J. van Lier, G. Zeeman, L. W. Huishoff Pol, Advanced Anaerobic Wastewater Treatment in the Near Future, Water Science and Technology, Vol. 35, No. 10, 1997, pp. 5-12, https://doi.org/10.1016/S0273-1223(97) 00222-9.

[12] G. L. Miller, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar, Analytical Chemistry, Vol. 31, 1959, pp. 426-428, https://doi.org/10.1021/ac60147a030.

[13] A. Sallam, A. Steinbüchel, *Clostridium sulfidigenes* sp. nov., A Mesophilic, Proteolytic, Thiosulfate- and Sulfur-reducing Bacterium Isolated from Pond Sediment, International of Journal Systematic and Evolutionary Microbiology, Vol. 59, 2009, pp. 1661-1665, https://doi.org/10.1099/ijs.0.004986-0.

[14] D. Evvyernie, S. Yamazaki, K. Morimoto, S. Karita, T. Kimura, K. Sakka, K. Ohmiya, Identification and Characterization of *Clostridium paraputrificum* M-21, a Chitinolytic, Mesophilic and Hydrogen-producing Bacterium, Journal of Bioscience and Bioengineering, Vol. 89, No. 6, 2000, pp. 596-601, https://doi.org/10.1016/s1389-1723(00)80063-8.

[15] C. C. Wang, C. W. Chang, C. P. Chu, D. J. Lee, B. V. Chang, C. S. Liao, Producing Hydrogen from Wastewater Sludge by *Clostridium bifermentans*, Journal of Biotechnology, Vol. 102, No. 1, 2003, pp 83-92, https://doi.org/10.1016/s0168-1656(03)00007-5.

[16] L. Beckers, J. Masset, C. Hamilton, F. Delvigne, D. Toye, M. Crine, S. Hiligsmann, Investigation of the Links between Mass Transfer Conditions, Dissolved Hydrogen Concentration and Biohydrogen Production by the Pure Strain *Clostridium butyricum* CWBI1009, Biochemical Engineering Journal, Vol. 98, 2015, pp. 18-28, https://doi.org/10.1016/j.bej.2015.01.008.

[17] H. Wang, S. Ma, H. Bu, Fermentative Hydrogen Production by Newly Isolated *Clostridium perfringens* ATCC 13124, Journal of Renewable and Sustainable Energy, Vol. 6, No. 1, 2014, pp. 013130, https://doi.org/10.1063/1.4863085.

[18] T. H. Vui, H. H. Diem, D. P. Ngoc, M. T. Hanh, T. V. B. Ha, A. N. Tien, Hydrogen Production by Newly Isolated *Clostridium* Species from Cow Rumen in Pure- and Co-cultures on a Broad Range of Carbon Sources, AIMS Energy, Vol. 6, 2018, pp. 846e6, https://doi.org/10.3934/ energy.2018.5.846.

[19] J. Wang, Y. Yin, A *Clostridium* Species for Fermentative Hydrogen Production: An Overview, International Journal of Hydrogen Energy, Vol. 46, No. 70, 2021, pp 34599-34625, https://doi.org/10.1016/j.ijhydene.2021.08.052.