Asia-Pacific Journal of Science and Technology: Volume: 30. Issue: 01. Article ID.: APST-30-01-05. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by Research Department,
Khon Kaen University, Thailand

Sustainable hydrogen production from oil palm trunk biomass: Optimization of hydrolysis and fermentation conditions with *Clostridium beijerinckii* PS-3

Pongsak Noparat[1,*] and Jiravut Seengenyoung[2]

[1]Program in Natural Resources and Environment, Faculty of Science and Technology, Suratthani Rajabhat University, Suratthani, Thailand
[2]ASEAN Biological Engineering Society, Songkhla, Thailand
[*]Corresponding author: pongsak@sru.ac.th

Received 24 October 2023
Revised 22 April 2024
Accepted 9 July 2024

Abstract

The study aimed to advance biohydrogen production by leveraging oil palm trunk (OPT) hydrolysate as a sustainable feedstock for *Clostridium beijerinckii* PS-3. Through a controlled hydrolysis process, OPT was treated with sulfuric acid (H_2SO_4) concentrations from 0.05% to 3.0% (v/v) over reaction times of 5 to 20 minutes at 121ºC. The optimal hydrolysis condition, determined to be 0.1% H_2SO_4 for 15 minutes, produced a hydrolysate rich in fermentable sugars, with a total concentration of 24.02 g/L—comprising 17.04 g/L xylose, 5.11 g/L glucose, 1.87 g/L arabinose, and 2.59 g/L acetic acid. To maximize hydrogen yield, response surface methodology (RSM) and central composite design (CCD) were employed, identifying peptone, iron, and nickel as critical elements influencing production efficiency. Optimal concentrations of 3.50 g/L peptone, 0.80 mM iron, and 0.80 mM nickel were predicted to yield a maximum of 2685 mL/L hydrogen. Experimental validation under refined conditions of 3.25 g/L peptone, 0.63 mM Fe^{2+}, and 0.74 mM Ni^{2+}, at an initial pH of 6.3 and 30ºC, surpassed predictions, achieving 2832 mL/L cumulative hydrogen and a yield of 236 mL H_2/g total sugar—an 11% increase over conventional media. Triplicate trials confirmed the consistency and robustness of OPT hydrolysate as a substrate for fermentative hydrogen production by *C. beijerinckii* PS-3. The substantial yield improvement illustrates the potential of OPT hydrolysate as a viable, low-cost resource for biohydrogen, emphasizing the importance of tailored hydrolysis and medium optimization in enhancing bioenergy production processes. Such advancements affirm the role of agricultural waste valorization in achieving economically feasible and environmentally sustainable biofuel solutions.

Keywords: biofuel, oil palm trunk hydrolysate, *Clostridium beijerinckii*, renewable energy

1. Introduction

Fermentative hydrogen production presents a transformative opportunity in clean energy development by leveraging dark fermentation, where hydrogen-producing bacteria metabolize organic substrates to yield hydrogen gas—a high-energy, clean fuel source [1]. As the urgency to counter climate change and reduce greenhouse gas emissions intensifies, a pivot to renewable energy sources has become essential, positioning hydrogen as a particularly versatile and efficient energy carrier capable of addressing diverse global energy demands with minimal environmental impact [2]. Unique among fuels, hydrogen combusts to produce only water vapor, offering an environmentally benign solution applicable across multiple sectors, including transportation and industry, which are traditionally heavy polluters [3]. Within this context, Thailand's substantial agricultural output, especially its oil palm industry, generates vast amounts of waste material with untapped energy potential. The country produces approximately 8 million tonnes of oil palm trunks annually, spread across 25 provinces, predominantly in the south. In 2018 alone, the palm oil yield reached 15.53 million tons, with fresh fruit bunches (FFB) contributing 5.35 million tons [4].

Nonetheless, despite the significant scale, much of the oil palm trunk residue remains abandoned after replanting, highlighting an inefficiency that could be strategically transformed into economic value and sustainable energy through biohydrogen production in Thailand. Oil palm trunks (OPT) contain substantial amounts of cellulose (31.28-42.85%), hemicellulose (19.73-25.56%), lignin (10.74-18.47%), protein (1.63-2.25%), fat (1.60-1.83%), ash (1.12-1.35%), and trace minerals (0.01-0.40%) [5], with hemicellulose standing out as a critical component for hydrogen production. Efficient extraction of hemicellulose through methods such as dilute-acid hydrolysis [6], steam explosion [7], and autohydrolysis [8] supports the conversion of OPT into biohydrogen, presenting a dual benefit: sustainable energy generation and an optimized approach to agricultural waste management. The potential to repurpose OPT for clean energy not only provides an economically advantageous pathway but also reinforces Thailand's commitment to environmental stewardship and renewable energy innovation.

The acid hydrolysis of OPT biomass focuses on effectively solubilizing its hemicellulosic fraction, leveraging both short residence times and high conversion efficiencies to produce fermentable sugars critical for bioenergy applications [9]. Although bioenergy production from lignocellulosic biomass has made commercial strides globally, it remains underexploited in regions such as Thailand, presenting a compelling opportunity for OPT-derived biohydrogen production [10]. OPT hydrolysate, with its high concentrations of hemicellulose and cellulose, provides an ideal substrate for microbial fermentation, specifically targeting hydrogen production. *Clostridium beijerinckii* PS-3, a robust rod-shaped and gram-positive bacterium with distinctive metabolic capabilities, has been identified as a particularly promising microorganism for this process. Known for its ability to metabolize a range of carbon sources, including sugars and organic acids, *C. beijerinckii* PS-3 converts these substrates into hydrogen gas, highlighting its potential in sustainable energy generation [11]. Thus, the current research not only explores the biohydrogen potential of OPT, transforming agricultural waste into a renewable energy source, but also aligns with broader economic and environmental objectives by adding value to otherwise discarded residues.

OPT hydrolysate offers a substantial reservoir of fermentable carbohydrates, enabling *C. beijerinckii* PS-3 to achieve high levels of hydrogen production—a trait that has been the focus of extensive studies due to the organism's resilience and adaptability under various environmental conditions. The metabolic functions of anaerobic microorganisms such as *C. beijerinckii* PS-3 are further supported by essential trace elements such as nickel (Ni^{+2}) and iron (Fe^{+2}), which constitute the active sites of hydrogenase enzymes crucial for hydrogen production [12]. Through an optimized use of OPT hydrolysate and a strategic understanding of the role of trace elements in microbial hydrogen pathways, this study offers significant contributions toward advancing biohydrogen as a viable clean energy source, leveraging agricultural by-products in a way that promotes both renewable energy innovation and sustainable waste management.

This study aims to optimize hydrogen production using oil palm trunk hydrolysate as a substrate and *C. beijerinckii* PS-3 as the hydrogen-producing bacterium. By investigating key parameters such as pH, temperature, substrate concentration, and nutrient supplementation, the study seeks to enhance hydrogen yield and production efficiency. Optimizing this process holds significant potential for developing a sustainable and economically viable bioenergy production system based on renewable resources.

2. Materials and methods

2.1 Inocula and growth medium

C. beijerinckii PS-3, isolated from oil palm sap, was cultivated in a synthetic medium known to support hydrogen production [11]. The medium contained 10 g/L xylose with an initial pH of 7.0. The bacterium was grown in anaerobic cultures for 18 hours at 30ºC in serum bottles. Following cultivation, the cultures were stored at 4ºC as stock culture for up to one month. Before each experiment, 1 mL of the stock culture with an optical density (OD660) of 0.5 was transferred to 5 mL of fresh synthetic medium. To create anaerobic conditions, the serum bottles were flushed with nitrogen for 3 minutes and then incubated at 30ºC for 18 hours.

2.2 Acid hydrolysis of OPT

OPT were sourced from Surat Thani Province in Southern Thailand. The trunks were air-dried, milled to pass through a 0.5 mm screen, and stored in plastic bags at room temperature until use. Acid hydrolysis of OPT was conducted in an autoclave at 121ºC for 15 minutes using various concentrations of H_2SO_4 (0.05%, 0.1%, 0.5%, 1%, 2%, and 3% v/v) with a solid-to-liquid mass ratio of 1:10. The resulting hydrolysates from each condition were used as substrates for fermentative hydrogen production. The H_2SO_4 concentration that yielded the highest hydrogen accumulation was further investigated to assess its effect on reaction time, with reaction times of 5, 10, 15, 20, and 25 minutes being tested. After hydrolysis, the solid residues were filtered through a thin cloth. The pH of the hydrolysate was adjusted to 7 using 2.5 M $Ca(OH)_2$, and the remaining residue was removed by centrifugation at 2000xg for 15 minutes [6].

2.3 Experimental design

A response surface methodology (RSM) was used to optimize hydrolysis, employing a two-level, three-variable central composite design with 20 experiments. Three parameters were varied: peptone concentration (1-6 g/L), $FeSO_4 \cdot 7H_2O$ concentration (0.1-1.5 mM), and $NiCl_2 \cdot 6H_2O$ concentration (0.1-1.5 mM). A hydrolysate concentration of 60% (v/v) was selected to optimize hydrogen production. Each experiment was conducted in serum bottles containing 45 mL of hydrolysate inoculated with 5 mL of *C. beijerinckii* PS-3. The initial pH was adjusted to 6.3 using 2M NaOH or 2M HCl, and the cultures were incubated at 30ºC for 48 and 96 hours.

All experiments were performed in triplicate. Culture broth samples were taken from the serum bottles for analysis of pH, substrate, and end-products. Volumetric hydrogen production (mL/L) was calculated based on the total gas production rate and the concentration of hydrogen in headspace. A factorial central composite design (CCD) was applied to investigate the effects of peptone, Fe^{2+}, and Ni^{2+} concentrations on hydrogen production, with cumulative hydrogen production monitored throughout the batch experiment. The experimental process variables (peptone, iron, and nickel concentrations) used for hydrogen production are shown in Table 1. A quadratic model [13] was utilized to evaluate the optimization of the medium compositions.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \quad (1)$$

where Y = predicted response (hydrogen production); x_1, x_2 and x_3 = parameters; β_0 = offset term; β_1, β_2 and β_3 = linear coefficients; β_{11}, β_{22} and β_{33} = squared coefficients; and β_{12}, β_{23} and β_{13} = interaction coefficients. The response variable (Y_{H2}) was fitted using a predictive polynomial quadratic equation (1) to correlate the response variable to the independent variables [14]. The Y_{H2} values were regressed against peptone, iron, and nickel concentrations using Statistica (Statsoft, USA). The optimal conditions predicted by RSM were experimentally validated to confirm the model's accuracy. The experiment was conducted in a 120 mL serum bottle, with samples collected every 12 hours over a period of 96 hours to measure hydrogen production, substrate utilization, and fermentation end-products.

Table 1 Design of experiments ranges and levels for hydrogen production.

Independent variable	Symbol	Range and levels				
		-α	-1	0	1	α
Peptone (g/L)	X_1	1.00	2.01	3.50	4.99	6.00
$FeSO_4 \cdot 7H_2O$ (mM)	X_2	0.10	0.38	0.80	1.22	1.50
$NiCl_2 \cdot 6H_2O$ (mM)	X_3	0.10	0.38	0.80	1.22	1.50

2.4 Analytical methods

The biogas produced during fermentation was collected in the headspace of the serum bottles, and its hydrogen content was measured using a gas meter [15]. The culture broth was centrifuged at 10,000 x g for 10 minutes, and the end-products in the supernatant were analyzed using gas chromatography (GC). The injection port temperature was set to 230ºC. The GC program began with an initial temperature of 70ºC, held for 1 minute, followed by a ramp of 20ºC/min up to 180ºC, and then a 6-minute hold at 180ºC. The detector temperature was maintained at 250ºC [16]. Glucose, xylose, and arabinose concentrations were determined using high-performance liquid chromatography (HPLC) [17].

3. Results and discussion

3.1 Composition of OPT hydrolysate

The composition of acid hydrolysate derived from OPT biomass is predominantly characterized by high concentrations of xylose and glucose, alongside trace amounts of arabinose, as evidenced by the measurements across varying H_2SO_4 concentrations (Table 2). Detailed compositional analysis indicates that the hydrolysate contains various components such as xylose, glucose, arabinose, acetic acid, hydroxymethylfurfural (HMF), furfural, and total sugars, with the sugar concentrations quantified under H_2SO_4 concentrations ranging from 0.05% to 3.0%. Xylose, primarily sourced from the hemicellulosic fraction, reached its highest concentration of 17.02 g/L at an H_2SO_4 concentration of 0.1% with a reaction time of 15 minutes. The increment in H_2SO_4 concentration from 0.05% (yielding 8.02 g/L of xylose) to 0.1% significantly enhanced xylose extraction, suggesting an optimal acidic environment for the effective breakdown of hemicellulose. Conversely, further elevation of acid concentration beyond 0.1% did not significantly affect glucose levels in the hydrolysate, indicating a threshold beyond which higher acidity does not benefit glucose release. Moreover, the reduction in xylose concentration at H_2SO_4 concentrations of 1.0% and 2.0%, followed by a slight recovery at 3.0%, implies a

complex interaction between acid concentration and sugar stability, where excessive acid may lead to sugar degradation or alter the reaction dynamics unfavorably. Thus, the observed data accentuate the critical need to fine-tune acid concentrations to optimize sugar recovery for efficient biohydrogen production from OPT, balancing the hydrolytic effectiveness against potential sugar losses at higher acid levels.

Acetic acid, an inhibitory substance frequently generated during the hydrolysis of hemicellulose [18], presents a significant concern in the context of OPT hydrolysate, with concentrations documented in the range of 2.37 to 2.76 g/L across various H_2SO_4 concentrations from 0.05% to 3.0% (Table 2). These acetic acid levels do not exhibit variations corresponding to changes in the concentration of H_2SO_4, a finding that diverges from the patterns observed by Wang et al., who reported a dependency on acid concentration [9]. Acetic acid levels exceeding 4 g/L begin to pose inhibitory risks to microbial processes, as concentrations up to 10 g/L can penetrate cell membranes, subsequently disrupting microbial metabolism by creating an acidic intracellular environment that hampers enzyme activity and energy production [19]. However, with the maximum acetic acid concentration in the OPT hydrolysate documented at 2.76 g/L, the levels remain well below the threshold known to inhibit microbial activity. The maintenance of acetic acid concentrations below inhibitory levels significantly enhances the suitability of OPT hydrolysate as a substrate for microbial fermentation processes in biohydrogen production, ensuring that metabolic efficiency is not compromised and thereby facilitating more robust and reliable bioenergy outputs.

Table 2 Composition of OPT hydrolysate obtained at a reaction time of 15 min under different concentrations of H_2SO_4.

H_2SO_4 (%)	Concentration (g/L)						
	Xylose	Glucose	Arabinose	Acetic acid	HMF	Furfural	Total sugar
0.05	8.02 ± 0.08^{a}	4.73 ± 0.03^{a}	1.68 ± 0.02^{a}	2.37 ± 0.12^{a}	0.44 ± 0.01^{a}	0.08 ± 0.01^{a}	14.43 ± 0.13
0.1	17.02 ± 0.12^{d}	5.08 ± 0.02^{b}	1.85 ± 0.01^{b}	2.53 ± 0.02^{ab}	0.65 ± 0.02^{b}	0.13 ± 0.01^{b}	23.95 ± 0.15
1.0	13.95 ± 0.16^{b}	4.15 ± 0.09^{e}	2.70 ± 0.01^{e}	2.65 ± 0.20^{ab}	0.72 ± 0.01^{c}	0.15 ± 0.01^{bc}	20.80 ± 0.26
2.0	14.53 ± 0.24^{c}	3.70 ± 0.03^{c}	2.37 ± 0.04^{c}	2.73 ± 0.21^{b}	0.85 ± 0.01^{d}	0.18 ± 0.01^{c}	20.60 ± 0.31
3.0	14.98 ± 0.18^{c}	4.35 ± 0.11^{d}	2.60 ± 0.03^{d}	2.76 ± 0.05^{c}	1.14 ± 0.03^{e}	0.25 ± 0.02^{d}	21.93 ± 0.32

Different letters indicate significant differences among treatments by the Duncan test ($p < 0.005$)

Optimizing the hydrolysis of OPT for economic viability and to minimize equipment corrosion necessitates the use of lower acid concentrations and shorter reaction times. An optimal H_2SO_4 concentration of 0.1% at 121°C for 15 minutes yielded the highest total sugar concentration of 23.95 g/L, establishing a promising foundation for further investigation to discern the impact of reaction time variations on hydrolysis efficiency. Under these conditions, peak xylose and glucose concentrations were 17.04 g/L and 5.11 g/L, respectively, as shown in Table 3. Extending the reaction time beyond 15 minutes, however, resulted in reduced concentrations of these sugars, suggesting degradation processes that could be exacerbated by prolonged exposure to acid, while concurrently, acetic acid levels increased, potentially introducing inhibitory effects on subsequent microbial fermentation processes. Specifically, arabinose reached its maximum concentration of 2.10 g/L at a longer reaction time of 25 minutes, indicating that different sugars respond variably to the hydrolysis conditions. The established optimal parameters for high-yield hydrolysate production include a reaction time of 15 minutes, which produced a hydrolysate containing desirable sugar levels—17.02 g/L of xylose and 5.08 g/L of glucose—with a moderate acetic acid concentration of 2.53 g/L. Such strategic optimization of hydrolysis conditions indicates the potential of mild acid hydrolysis to efficiently process OPT into a viable substrate for biohydrogen production, thereby balancing cost-effectiveness, operational safety, and sugar recovery for enhanced sustainability in energy applications.

The hydrolysis of OPT biomass under controlled conditions of H_2SO_4 concentrations and reaction times provides significant insights into maximizing fermentable sugar yields. Detailed compositions of the OPT hydrolysate, including xylose, glucose, arabinose, acetic acid, 5-hydroxymethylfurfural (HMF), furfural, and total sugars, are systematically documented in Table 3. The process demonstrated a peak xylose concentration of 17.04 g/L at an optimal H_2SO_4 concentration of 0.1% over a 15-minute reaction period. The increase in xylose was substantial when the acid concentration was adjusted from 0.05% to 0.1%, suggesting an efficient breakdown of hemicellulose under mild acidic conditions. Nevertheless, elevating the H_2SO_4 concentration further to 1.0%, 2.0%, and 3.0% did not enhance xylose output, indicating a threshold beyond which additional acid does not facilitate further sugar release. Relatedly, glucose concentration also plateaued at 5.11 g/L under the same optimal conditions, with no further increments at higher acid concentrations. Arabinose, some sugar less readily released from arabinoxylans, peaked at 2.10 g/L for a reaction time of 25 minutes, revealing slower hydrolysis kinetics relative to xylose and glucose. Acetic acid levels, which can impede microbial metabolism if elevated, increased with reaction time but were unaffected by variations in acid concentration, remaining stable across the studied range. The low concentrations of HMF and furfural across all conditions suggest minimal sugar degradation, enhancing the hydrolysate's suitability for bioenergy applications. The empirically determined optimal hydrolysis condition of 0.1% H_2SO_4 at 121°C for 15 minutes, which yielded 17.04 g/L xylose, 5.11 g/L glucose, and 2.59 g/L acetic acid, illustrates the profound influence of acid concentration and reaction time on the hydrolysate's compositional quality. Such findings suggest that carefully calibrated hydrolysis conditions are critical for preparing a hydrolysate

that is both rich in sugars and low in inhibitors, ideal for fermentative hydrogen production using microorganisms, such as *Clostridium beijerinckii* PS-3, thus advancing the development of sustainable bioenergy solutions.

Table 3 Composition of OPT hydrolysate obtained at H_2SO_4 of 0.1% under different reaction times.

Reaction time (min)	Concentration (g/L)						
	Xylose	lucose	Arabinose	Acetic acid	HMF	Furfural	Total sugar
5	5.09 ± 0.03^{a}	3.59 ± 0.05^{a}	1.86 ± 0.10^{ab}	1.95 ± 0.12^{a}	0.35 ± 0.01^{a}	0.03 ± 0.01^{a}	10.54 ± 0.18
10	12.38 ± 0.02^{b}	4.25 ± 0.02^{b}	1.75 ± 0.20^{a}	2.37 ± 0.01^{b}	0.66 ± 0.01^{b}	0.12 ± 0.01^{b}	18.38 ± 0.24
15	17.04 ± 0.07^{d}	5.11 ± 0.12^{d}	1.87 ± 0.08^{ab}	2.59 ± 0.09^{bc}	0.68 ± 0.01^{bc}	0.16 ± 0.01^{bc}	24.02 ± 0.27
20	16.85 ± 0.09^{d}	5.09 ± 0.17^{a}	2.04 ± 0.04^{b}	2.68 ± 0.07^{c}	0.71 ± 0.01^{c}	0.18 ± 0.02^{cd}	23.98 ± 0.30
25	14.20 ± 0.10^{c}	4.90 ± 0.08^{c}	2.10 ± 0.12^{b}	2.62 ± 0.04^{c}	0.75 ± 0.01^{d}	0.22 ± 0.01^{d}	21.20 ± 0.30

Different letters indicate significant differences among treatments by the Duncan test ($p < 0.005$)

3.2 Optimization reaction for hydrogen production

The hydrogen production results obtained ranged from 875 to 2685 mL H_2/L (Table 4). The highest hydrogen production of 2685 mL H_2/L was achieved under moderate concentrations of peptone, iron, and nickel (run 19). A regression analysis was conducted to examine the data and understand the relationship between the nutrient variables and hydrogen production. The data presented in Table 4 were fitted to a quadratic equation as follows:

$$Y_{H2} = +2597.34 + 46.60X_1 - 139.63X_2 - 207.96X_3 - 22.88X_1X_2 - 19.87X_1X_3 + 81.38X_2X_3 - 495.84X_1^2 - 394.19X_2^2 - 501.49X_3^2 \quad (2)$$

Where Y_{H2} represents the predicted hydrogen production, X_1, X_2, and X_3 represent the nutrient variables peptone, iron, and nickel concentrations, respectively, and +2597.34, +46.60, -139.63, -207.96, -22.88, -19.87, +81.38, -495.84, -394.19, and -501.49 are the regression coefficients. This quadratic equation enables the evaluation of nutrient concentration optimization for enhanced hydrogen production. By analyzing the regression coefficients, we can assess the significance and magnitude of the effects of each nutrient variable on hydrogen production. The data in Table 4 provide valuable insights into the relationship between nutrient concentrations and hydrogen production. Through regression analysis, the optimal combination of nutrient concentrations can be identified to maximize hydrogen production.

Table 4 Central composite design matrix defining peptone, iron, and nickel concentration and results on hydrogen production, where X_1= peptone (g/L), X_2= $FeSO_4 \cdot 7H_2O$ (mM), and X_3= $NiCl_2 \cdot 6H_2O$ (mM).

Runs	Variables			Cumulative H_2 production (mL/L)
	Peptone	$FeSO_4 \cdot 7H_2O$	$NiCl_2 \cdot 6H_2O$	
1	1.00	0.80	0.80	1155 ± 32
2	4.99	0.38	0.38	1760 ± 26
3	2.01	0.38	0.38	1494 ± 18
4	6.00	0.80	0.80	1270 ± 22
5	3.50	0.80	0.80	2558 ± 49
6	2.01	1.22	1.22	875 ± 17
7	3.50	1.50	0.80	1173 ± 34
8	3.50	0.80	0.80	2575 ± 28
9	3.50	0.80	0.80	2560 ± 70
10	4.99	1.22	0.38	1280 ± 38
11	4.99	1.22	1.22	970 ± 21
12	2.01	0.38	1.22	938 ± 19
13	3.50	0.80	0.80	2650 ± 32
14	2.01	1.22	0.38	1245 ± 16
15	4.99	0.38	1.22	985 ± 12
16	3.50	0.10	0.80	1827 ± 15
17	3.50	0.80	0.10	1443 ± 18
18	3.50	0.80	1.50	950 ± 14
19	3.50	0.80	0.80	2685 ± 14
20	3.50	0.80	0.80	2550 ± 45

The RSM model exhibited a high determination coefficient ($R_2 = 0.99$), indicating that it explained 99% of the variability in the response. Additionally, the adjusted determination coefficient (adjusted $R_2 = 0.98$) showed a high significance of the model [18]. The analysis of variance (ANOVA) analysis confirmed the high importance of the quadratic regression model, as evidenced by the Fisher's F-test yielding a deficient probability value ($P<0.0001$). Furthermore, the lack of fit model was insignificant ($P=0.0521$), suggesting that the model adequately represented the data. The coefficient of variation (CV) value, which was relatively low at 5.94%, indicated the experimental results' high precision and reliability. The equation was set to zero for the corresponding variables to determine the optimum conditions for maximum hydrogen production yield. These calculations determined the optimal nutrient concentrations to be 3.25 g/L peptone, 0.63 mM Fe^{2+}, and 0.74 mM Ni^{2+}. The RSM model, with

its high determination coefficient, significant ANOVA results, and low coefficient of variation, provides strong evidence for the accuracy and reliability of the optimized conditions.

The optimization of hydrogen production, achieving a maximum value of 2794 mL/L, highlights the effectiveness of variable manipulation, as detailed through the analytical response surface and contour plots derived from Equation (2). In these graphical representations, while maintaining one variable at its optimum, the other two were adjusted within the experimental parameters (Figure 1A, 1B, 1C), facilitating a thorough understanding of the factors driving hydrogen yields. Such plots are instrumental in discerning the complex relationships among variables that influence hydrogen production. Previous research corroborates the pivotal roles of Fe^{2+} and Ni^{2+} in this process, with the metals acting as essential cofactors in the enzymatic mechanisms that govern biohydrogen production [20]. Hydrogenases, the enzymes central to hydrogen production, are differentiated into [Ni-Fe] and [Fe-Fe] types based on the metallic composition at their active sites [21]. The [Ni-Fe] hydrogenases, reliant on nickel and iron, are notably sensitive to the concentrations of these metals, which significantly impact the yields of fermentative hydrogen [22]. Extensive studies have proved that iron supplementation in batch tests markedly enhances hydrogen production [23]. Moreover, maintaining a controlled increase in Ni^{2+} concentration up to 0.2 mg/L at 35°C not only augments hydrogen production but also preserves glucose degradation efficiency. Research has shown that adjustments in Ni^{2+} levels can trigger metabolic pathway shifts, indicating a potential regulatory mechanism that could be leveraged to fine-tune hydrogen production [24, 25]. These insights suggest that strategic control over metal ion concentrations could optimize enzyme activity, thereby enhancing the efficiency and sustainability of biohydrogen production processes.

The results of the multiple linear regression analysis indicated that the concentrations of peptone, Fe^{2+}, and Ni^{2+} significantly influenced hydrogen production. Among the variables, it was evident that the iron concentration (X_2) and nickel concentration (X_3) had a significant effect on hydrogen production ($P<0.05$). Furthermore, both the linear (X_2, X_3, X_1^2, X_2^2, X_3^2) and quadratic (X_2X_3) terms of the iron and nickel concentrations were also found to be significant ($P<0.05$). The coefficient estimates presented in Table 5 provide insights into the magnitude and direction of the effects of the variables on hydrogen production. The intercept term represents the baseline hydrogen production, while the coefficient estimates for X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, and X_1X_3 reflect the contributions of each variable to overall hydrogen production. Based on the significance of the regression coefficients, it can be concluded that the concentrations of iron and nickel play a crucial role in enhancing hydrogen production. These findings emphasize the importance of optimizing the supplementation of Fe^{2+} and Ni^{2+} to maximize fermentative hydrogen production.

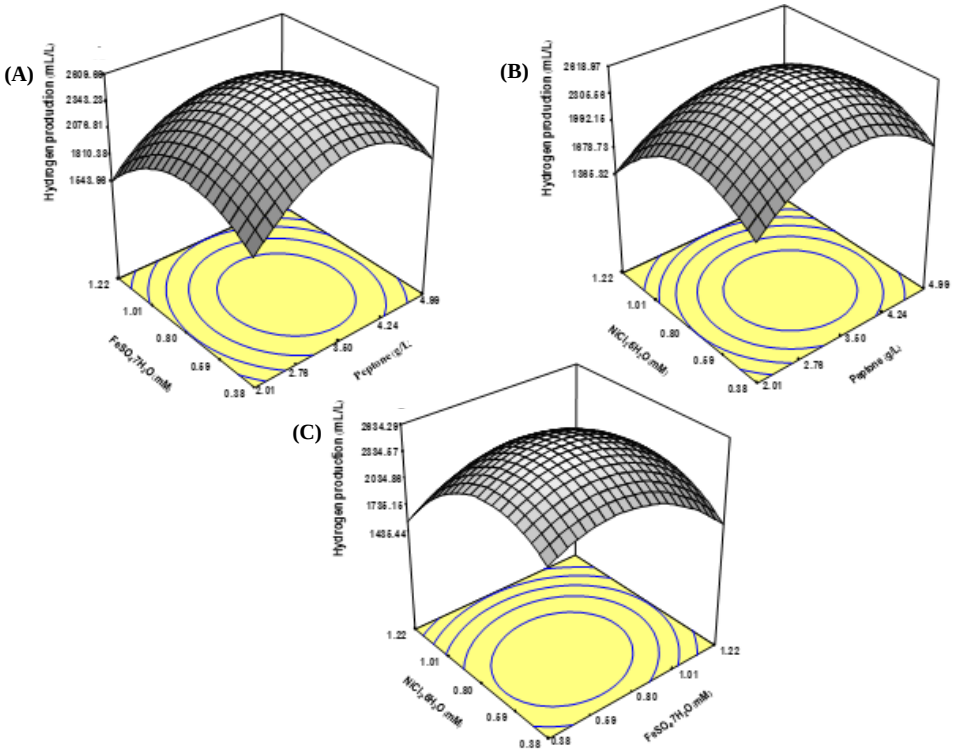

Figure 1 Response surface plot of hydrogen production by *Clostridium beijerinckii* PS-3 as a function of fixed $NiCl_2{\cdot}6H_2O$ at 0.80 mM (A), fixed $FeSO_4{\cdot}7H_2O$ at 0.80 g/L (B), and fixed peptone at 3.50 g/L (C).

Table 5 Coefficient estimates for a multiple linear regression.

Factor	Hydrogen production (Y_{H2})	
	Coefficient estimate	Probability
Intercept	2597.34	-
X_1	46.60	0.1091
X_2	-139.63	0.0004*
X_3	-207.96	<0.0001*
X_1^2	-495.84	<0.0001*
X_2^2	-394.19	<0.0001*
X_3^2	-501.49	<0.0001*
X_1X_2	-22.88	0.5237
X_1X_3	-19.87	0.5785
X_2X_3	81.38	0.0406*

* Significant level at 99%

3.3 Confirmation experiment

This study demonstrates that the optimal conditions for enhanced hydrogen production by *Clostridium beijerinckii* PS-3 are 3.50 g/L of peptone, 0.80 mM of iron, and 0.80 mM of nickel. According to the RSM model, this combination resulted in a predicted hydrogen production of 2685 mL H_2/L. Three replicates were conducted under these optimized conditions to validate the statistical experiments and further enhance our understanding of hydrogen production. The experimental results (Table 6) closely aligned with the predicted values, showing a measured hydrogen production of 2832 mL H_2/L. Furthermore, total sugar utilization reached 94%, indicating an efficient conversion of the available sugars in the hydrolysate. When comparing hydrogen production using OPT hydrolysate (Table 6, run 1) and the synthetic medium (Table 6, run 19), the hydrogen production from the optimized hydrolysate was significantly higher at 1245 mL H_2/L compared to 2574 mL H_2/L achieved with the synthetic medium alone. This highlights the potential of utilizing OPT hydrolysate as a sustainable substrate for enhanced hydrogen production. Notably, the hydrogen production achieved in this study using *Clostridium beijerinckii* PS-3 from OPT hydrolysate surpasses the hydrogen production reported for *Clostridium* strain BOH3 using fruit waste (510 mL H_2/L) [26].

Table 6 Confirmation of experimental design

Runs	Conditions	Peptone (g/L)	Fe^{2+} (mM)	Ni^{2+} (mM)	H_2 (mL/L)	Ethanol (mM)	Acetic acid (mM)	Butyric acid (mM)	Propionic acid (mM)
-	Optimal*	3.25	0.63	0.74	2832 ± 75^{e}	23.5 ± 0.9^{c}	5.2 ± 0.2^{b}	31.3 ± 1.1^{d}	2.6 ± 0.1^{c}
19	Medium	3.50	0.80	0.80	2574 ± 54^{d}	21.2 ± 1.1^{c}	6.7 ± 0.4^{c}	28.3 ± 0.6^{c}	2.4 ± 0.3^{bc}
6	Worst	2.01	1.22	1.22	750 ± 13^{a}	15.2 ± 0.5^{a}	3.4 ± 0.1^{a}	16.5 ± 0.5^{a}	0.8 ± 0.1^{a}
16	Endo	3.50	0.10	0.80	1986 ± 48^{c}	17.3 ± 0.8^{ab}	4.5 ± 0.5^{bc}	23.6 ± 1.2^{b}	1.6 ± 0.2^{ab}
1	Control	1.00	0.80	0.80	1245 ± 35^{b}	18.3 ± 1.2^{b}	4.2 ± 0.2^{ab}	24.9 ± 1.7^{b}	2.1 ± 0.1^{bc}

*Based on hydrogen production.
Different letters indicate significant differences among treatments by the Duncan test ($p < 0.005$)

Table 6 summarizes the results from the 48-hour confirmation experiments. We conducted three different runs to confirm the experimental design and evaluate the hydrogen production and fermentation end products under various conditions. The optimal conditions, represented by Run 19, consisted of 3.25 g/L peptone, 0.63 mM Fe^{2+}, and 0.74 mM Ni^{2+}. Under these conditions, the mean hydrogen production was 2832 ± 75 mL/L. Additionally, the concentrations of ethanol, acetic acid, butyric acid, and propionic acid were 23.5 ± 0.9 mM, 5.2 ± 0.2 mM, 31.3 ± 1.1 mM, and 2.6 ± 0.1 mM, respectively. These results confirm the high hydrogen production achieved under the optimized conditions. Comparatively, Run 1 (control) represented the lowest hydrogen production at 1245 ± 35 mL/L. The control run serves as a reference point for evaluating the improvement achieved through optimization. Run 6, conducted under the worst conditions with 2.01 g/L peptone, 1.22 mM Fe^{2+}, and 1.22 mM Ni^{2+}, produced 750 ± 13 mL/L hydrogen production. Run 16, conducted of 3.50 g/L peptone, 0.10 mM Fe^{2+}, and 0.80 mM Ni^{2+}, exhibited a hydrogen production of 1986 ± 48 mL/L. Overall, the results from the confirmation experiments align well with the predicted values, confirming the validity and reliability of the optimized conditions. The optimal conditions (Run 19) achieved the highest hydrogen production, with a mean value of 2832 mL/L, demonstrating the effectiveness of the designed experiments and the significance of the nutrient variables in enhancing hydrogen production.

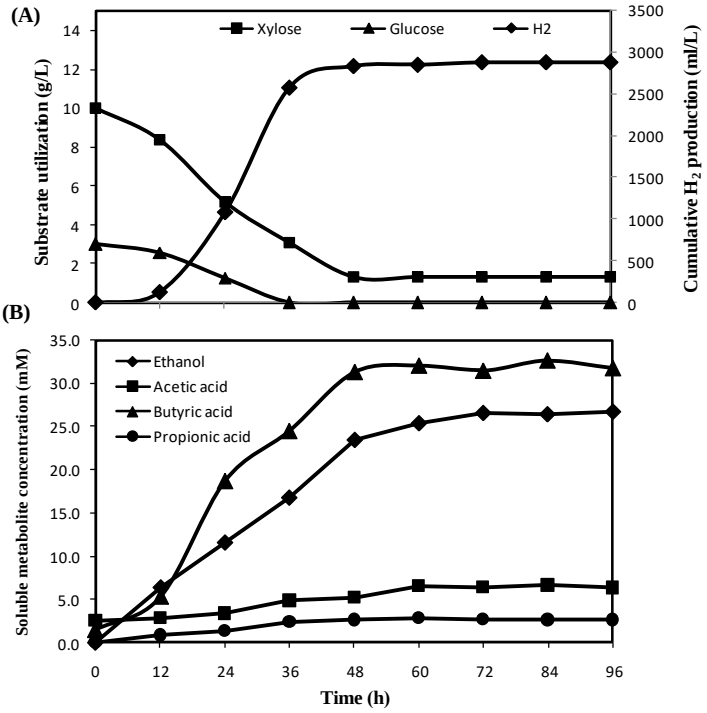

Figure 2 Cumulative hydrogen production, substrate utilization in the confirmation experiment at optimum condition (A), and soluble metabolites (B) using *Clostridium beijerinckii* PS-3.

The study indicates that hydrogen fermentation is accompanied by the production of volatile fatty acids, with butyric acid, acetic acid, and ethanol as the primary soluble metabolites, along with trace levels of propionic acid. The presence of butyric and acetic acids is particularly notable, as they are markers of efficient hydrogen fermentation, underscoring a predominance of butyrate-type fermentation pathways, which are essential for maximizing hydrogen yield [27]. A comparison with other studies using OPT hydrolysate as a substrate for hydrogen production reveals that the optimized process in this study achieved a significantly higher hydrogen yield of 236 mL H_2/g total sugar, compared to 189 mL H_2/g total sugar in previous reports [28]. Observations of the incubation process further clarify this efficiency, with hydrogen production and substrate utilization accelerating notably between 6 and 36 hours—moving from the lag phase to peak activity—before reaching equilibrium at 48 hours (Figure 2A, 2B). The hydrogen yield obtained with *C. beijerinckii* PS-3 under these optimized conditions closely matches, yet exceeds, previous findings with *E. harbinense* B49, indicating the potential of further refined conditions to enhance production efficiency even more [28]. This promising outcome suggests that exploring the underlying metabolic pathways in OPT hydrolysate fermentation could yield additional insights into the mechanisms that facilitate hydrogen generation. Dark fermentation processes typically involve the breakdown of complex organic molecules into simpler carbohydrates such as glycerol, hexose, and pentose, which hydrogen-producing *Clostridium* species subsequently convert into hydrogen [29]. For practical implementation, economic feasibility and scalability considerations are vital, especially given the cost advantages of using OPT hydrolysate as a feedstock. By transforming a low-cost agricultural byproduct into a renewable energy source, OPT hydrolysate not only minimizes input expenses but also aligns with sustainable bioenergy practices.

4. Conclusions

Optimal conditions for maximizing hydrogen production were determined to be 3.25 g/L peptone, 0.63 mM Fe^{2+}, and 0.74 mM Ni^{2+}, resulting in a maximum yield of 2794 mL H_2/L. The close alignment of experimental outcomes with predicted values demonstrates the reliability and effectiveness of the applied optimization approach, underscoring its value in advancing fermentative hydrogen production from OPT hydrolysate. The strategic identification of such optimal conditions is critical, as it allows for substantial increases in hydrogen yield, improved process efficiency, and a more effective use of resources, ultimately enhancing the viability of hydrogen as a bioenergy source. Employing RSM facilitated a structured exploration of the parameter space, revealing key factors that most profoundly influence hydrogen production. Such an approach to optimization not only enhances the production process but also aligns with broader goals of sustainable and resource-efficient bioenergy generation. Leveraging OPT hydrolysate, a cost-effective and renewable feedstock, within an optimized fermentation framework highlights a promising pathway for developing scalable, environmentally sustainable

hydrogen production systems. These findings disclose the transformative potential of optimized fermentation processes to meet growing energy demands while supporting environmental objectives, positioning hydrogen production from agricultural byproducts as a feasible and impactful component of the renewable energy sector.

5. Acknowledgements

This work was supported by ASEAN Biological Engineering Society and support from the Environmental Science Laboratory, Program in Natural Resources and Environment, Faculty of Science and Technology, Suratthani Rajabhat University.

6. References

[1] Sinbuathong N, Sillapacharoenkul B. Dark fermentation of starch factory wastewater with acid- and base-treated mixed microorganisms for biohydrogen production. Int J Hydrogen Energy. 2021;46(31):16622-16630.
[2] Tokarska KB, Gillett, NP. Cumulative carbon emissions budgets consistent with 1.5˚C global warming. Nat Clim Change. 2018;8:296-299.
[3] Zhu X, Imtiaz Q, Donat F, Müller CR, Li F. Chemical looping beyond combustion – a perspective. Energ Environ Sci. 2020;13:772-804.
[4] DOAE. Production and marketing of oil palm in 2018 [Internet]. Bangkok: Department of Agricultural Extension; 2018 [cited 2023 Sep 15]. Available from: https://www.doae.go.th/en/category/press-release/.
[5] Noparat P, Prasertsan P, O-Thong S, Pan X. Sulfite pretreatment to overcome recalcitrance of lignocellulose for enzymatic hydrolysis of oil palm trunk. Energy Procedia. 2017;138:1122-1127.
[6] Noparat P, Prasertsan P, O-Thong S, Pan X. Dilute acid pretreatment at high temperature of oil palm trunk biomass for enzymatic hydrolysis. Energy Procedia. 2015;79:924-929.
[7] Nasir A, Chen HZ, Wang L. Novel single-step pretreatment of steam explosion and choline chloride to de-lignify corn stover for enhancing enzymatic edibility. Process Biochem. 2020;94:273-281.
[8] Fradinhoa P, Oliveira A, Dominguez H, Torres MD, Sousa I, Raymundo A. Improving the nutritional performance of gluten-free pasta with potato peel autohydrolysis extract. Innov Food Sci Emerg Technol. 2020;63:102374.
[9] Wang H, Xie H, Du H, Wang X, Liu W, Duan Y, et al. Highly efficient preparation of functional and thermostable cellulose nanocrystals via H_2SO_4 intensified acetic acid hydrolysis. Carbohydr Polym. 2020; 239:116233.
[10] Nimmanterdwong P, Chalermsinsuwan B, Piumsomboon P. Emergy evaluation of biofuels production in Thailand from different feedstocks. Ecol Eng. 2015;74:423-437.
[11] Noparat P, Prasertsan P, O-Thong S. Isolation and characterization of high hydrogen-producing strain *Clostridium beijerinckii* PS-3 from fermented oil palm sap. Int J Hydrogen Energy. 2011;36:14086-14092.
[12] Mohan V, Srikanth S. Regulatory function of divalent cations in controlling the acidogenic biohydrogen production process. RSC Adv. 2012;2:6576-6589.
[13] Box GEP, Behnken DW. Three level design for the study of quantitative variables. Technometrics. 1960;2:455-475.
[14] Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng. 2000;68:269-278.
[15] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. Int J Hydrogen Energy. 2008;33:1204-1214.
[16] Miyazaki K, Irbis C, Takada J, Matsuura A. An ability of isolated strains to efficiently cooperate in ethanolic fermentation of agricultural plant refuse under initially aerobic thermophilic conditions: Oxygen deletion process appended to consolidated bioprocessing (CBP). Bioresour Technol. 2008;99:1768-1775.
[17] Delgenes JP, Moletta R, Navarro JM. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehae*. Enzym Microb Tech. 1996;9:220-225.
[18] Reaño RL. Assessment of environmental impact and energy performance of rice husk utilization in various biohydrogen production pathways. Bioresour Technol. 2020;299:122590.
[19] Li W, Cheng C, Cao G, Ren N. Enhanced biohydrogen production from sugarcane molasses by adding Ginkgo biloba leaves. Bioresour Technol. 2020;298:122523.
[20] Yin Y, Wang J. Optimization of fermentative hydrogen production by Enterococcus faecium INET2 using response surface methodology. Int J Hydrogen Energy. 2019;33(23):6976-6984.
[21] Sun Y, He J, Yang G, Sun G, Sage V. A review of the enhancement of bio-hydrogen generation by chemicals addition. Catalysts. 2019;9:353.
[22] Mishra P, Krishnan S, Rana S, Singh L, Sakinah M, Wahid Z. Outlook of fermentative hydrogen production techniques: An overview of dark, photo and integrated dark-photo fermentative approach to biomass. Energy Strat Rev. 2019;24:27-37.

[23] ThaoVi LV, Salakkam A, Reungsang, R. Optimization of key factors affecting bio-hydrogen production from sweet potato starch. Energy Procedia. 2017;138:973-978.
[24] Engliman NS, Abdul PM, Wu SY, Jahim JM. Influence of iron (II) oxide nanoparticle on biohydrogen production in thermophilic mixed fermentation. Int J Hydrogen Energy. 2017;42:27482-27493.
[25] Gou CY, Guo JB, Lian J, Guo YK, Jiang ZS, Yue L, et al. Characteristics and kinetics of biohydrogen production with Ni^{2+} using hydrogen-producing bacteria. Int J Hydrogen Energy. 2015;40:161-167.
[26] Mahato RK, Kumar D, Rajagopalan G. Biohydrogen production from fruit waste by *Clostridium* strain BOH3. Renew Energ. 2020;153:1368-1377.
[27] Martinez-Burgosa WJ, Sydneyb EB, de Paula DR, Medeiros ABP, de Carvalho JC, Soccol VT, et al. Biohydrogen production in cassava processing wastewater using microbialconsortia: Process optimization and kinetic analysis of the microbial community. Bioresour Technol. 2020;309:123331.
[28] Xie GJ, Li BF, Wang Q, Ding J, Ren NQ. Ultrasonic waste activated sludge disintegration for recovering multiple nutrients for biofuel production. Water Res. 2016;93:55-64.
[29] Wang J, Yin Y. *Clostridium* species for fermentative hydrogen production: An overview. Int J Hydrogen Energy. 2021;46(70):34599-34625.