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Enhancing Biohydrogen Yield and Slaughterhouse Wastewater Treatment Efficiency through Microalgae and Bacterial Synergy

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Abstract

Slaughterhouse wastewater is highly rich in organic content. It carries an extremely high nutrient load when discharged into the environment, demonstrating that conventional treatment practices usually need to be more sustainable and extremely energy intensive. This research study probes various ways to integrate a microalgae-based platform to realize sustainable biohydrogen production, concurrently addressing critical wastewater treatment and renewable energy generation. A new two-stage cultivation method was developed using native wastewater bacteria and carbohydrate-rich microalgae, *Chlorella vulgaris* ESP 6. This method increased the efficiency of nutrient recovery and COD reduction compared to conventional single-stage systems. The microalgae, a key component in the process, efficiently converted the organic content of the wastewater into a carbohydrate-rich biomass, with a carbohydrate content of 48.8 ± 2.3%. This biomass was then fermented in the dark with *Clostridium butyricum* CGS5 to make biohydrogen after being treated with thermal acid. A pH control strategy through automatic operation was enforced, resulting in an impressive improvement in hydrogen production to 211 mL H_2/g volatile solids and a maximum productivity of 39.11 mL/L/h, both competitive with the current literature. This study points out a promising way of replacing conventional wastewater treatment with integrated nutrient removal and energy recovery and utilizing wastewater biomass rich in carbohydrates as a renewable feedstock for biohydrogen production. Overall, this is the highest hydrogen yield ever reported on slaughterhouse wastewater and may be highly important for further development in waste-to-energy applications.

Keywords

biohydrogen, microalgae, cultivation, wastewater, nutrient, renewable energy

1 Introduction

The escalating global population and rapid economic growth have significantly increased the energy demand, predominantly from fossil fuels. Historically, the combustion of fossil fuels has led to substantial greenhouse gas emissions, contributing to climate change and various environmental issues. As environmental concerns intensify, there is an urgent need to transition from traditional, non-renewable energy sources to cleaner, sustainable alternatives. This transition is not just a scientific endeavor, but a global imperative. Among these alternatives, hydrogen has emerged as a promising eco-friendly energy source, mainly due to its high energy density of approximately 120 MJ/kg and water being the only byproduct of its combustion [1].

The International Energy Agency (IEA) has projected that global hydrogen demand could exceed 500 MMT (million metric tons) by 2070, primarily driven by transportation needs [2]. Research has identified various microorganisms capable of producing hydrogen gas, with *Clostridium butyricum* recognized as a leading bacterium for natural hydrogen production via fermentation [3, 4]. This bacterium yields high amounts of hydrogen and demonstrates versatility in decomposing various organic pollutants, including food waste and agricultural residues.

In recent years, there has been a growing interest in utilizing algae for hydrogen production due to their rapid growth rates, high carbohydrate content, and natural ability to absorb carbon dioxide. Microalgae play a crucial

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role in wastewater purification by effectively removing pollutants and recovering nutrients, making them a viable feedstock for microbial biohydrogen production. Various algae species, such as *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Dunaliella salina*, have been studied, with hydrogen production rates ranging from 50 to 300 mL of hydrogen gas per gram of processed organic matter, depending on factors like algae strain, bacterial species, and operational conditions [5, 6].

Researchers have explored numerous methods to enhance biohydrogen production, including pre-treatment techniques (microwave and autoclave), co-digestion with additional substrates, and two-stage fermentation processes. Utilizing low-cost industrial byproducts as nutrient sources for microalgae cultivation presents significant potential for improving this third-generation biofuel's affordability and ecological sustainability.

The meat industry has seen increased production to meet rising food demands, leading to substantial quantities of slaughterhouse wastewater (SWW), rich in organic components like nitrogen and phosphorus. If untreated, SWW can cause environmental issues such as eutrophication. Research indicates that microalgae can effectively purify SWW while recovering nutrients for biohydrogen production. Studies [7, 8] highlight the potential of microalgae in this context achieving a maximum hydrogen output of 56.8 mL/g from dark fermentation of *C. vulgaris* and *S. obliquus*.

Efficient pre-treatment methods are critical for optimizing biohydrogen generation from microalgal biomass. Various thermal and chemical pre-treatment techniques have been examined, with microwave-assisted acid pre-treatment and alkaline treatment with sodium hydroxide showing significant improvements in hydrogen production by enhancing carbohydrate availability for fermentation.

Integrating microalgae for nitrogen removal and biohydrogen production is a novel approach that addresses wastewater treatment and renewable energy generation simultaneously. Studies have underscored the importance of selecting appropriate microalgae strains for system integration, as this optimization is crucial for achieving the dual goals of hydrogen production and nutrient recovery.

Wang and Wan [9] developed an integrated microalgae-based system for nutrient removal and biohydrogen generation from industrial wastewater, demonstrating improved treatment effectiveness and a sustainable biohydrogen source. Research on SWW as a microalgae growth medium has shown significant nutrient removal and potential for biohydrogen production, with studies emphasizing the environmental advantages of such systems [10].

Optimizing temperature, pH, and substrate concentrations is crucial for enhancing biohydrogen production from microalgal biomass. Nazarpour et al. [11] utilized response surface methodology to optimize fermentation conditions, revealing that ammonium addition significantly improved yields. Sharma et al. [12] found that substrate concentration increases hydrogen production rates, although excessive amounts may lead to inhibition.

The economic and environmental sustainability of microalgae-based systems is increasingly recognized, with recent analyses highlighting their cost-effectiveness and ecological advantages. Sangma and Chalie-u [13] conducted studies on life cycle assessment (LCA) of microalgae systems utilizing wastewater for biohydrogen production, demonstrating significant greenhouse gas emissions savings compared to traditional methods. Gurreri et al. [14] emphasized balancing financial and ecological sustainability in microalgae applications.

System integration is vital for enhancing microalgae-based wastewater treatment and biohydrogen production [15]. A recent research has investigated various integration methods to improve performance, with hybrid systems promising reduced operational costs and enhanced efficiency. Selecting high-productivity microalgae strains is essential for effective wastewater purification and biohydrogen production [16], with studies indicating that genetically modified strains may further enhance hydrogen production [17].

Scaling microalgae-based systems from research to industrial levels presents economic viability and operational capacity challenges. Techno-economic analyses have identified potential cost reductions through improved system structure and operational status [18–20]. Environmental sustainability remains a significant factor in implementing these systems, with studies revealing substantial reductions in greenhouse gas emissions and environmental impact compared to conventional methods [21, 22].

The potential of microalgae-based systems in treating wastewater and generating renewable energy as biohydrogen is immense [23, 24]. Their unique ability to assimilate nutrients and mitigate organic loads not only presents significant economic and environmental benefits but also opens up a promising avenue for future research and development in the quest for sustainable energy solutions.

2 Materials and methods

Fig. 1 shows the process flow of an innovative two-stage microalgae-bacteria treatment system, wholly integrated with dark fermentation for biohydrogen production, focusing on SWW remediation and biomass conversion. The detailed processes are structured to illustrate wastewater processing through several treatment stages, beginning with raw wastewater intake and ending with produced hydrogen and different volatile fatty acids.

The process commences with the injection of the SWW, which activates the extensive treatment system. The SWW undergoes a bio-restorative method utilizing a dual-remedial system with microalgae (*C. vulgaris* ESP 6) and native bacteria. SWW remediation is carried out in a photobioreactor system with a 1 L capacity, inoculated with biomass at a concentration of 0.3 g/L *C. vulgaris* ESP 6. The effectiveness of *C. vulgaris* ESP 6 culture in biomass production and nutrient extraction was evaluated at various sewage concentrations: 25%, 50%, 75%, and 100%. Comparable conditions were established for microalgal preculture. Systematic sampling measured biomass production, carbohydrate levels, and chemical oxygen demand (COD), total organic carbon (TOC) total nitrogen (TN), and total phosphorus (TP) concentrations, total solids (TS) and suspended solid (SS).

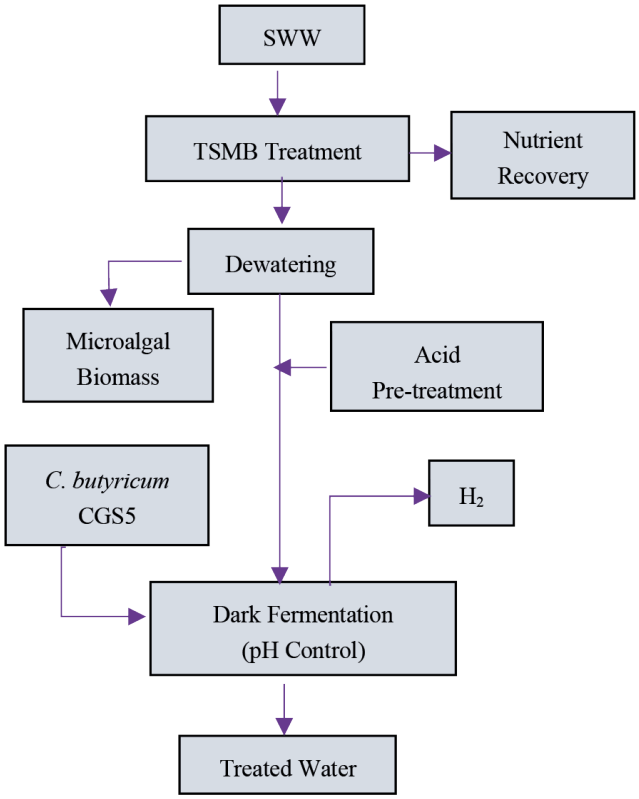


Fig. 1 Two-stage SWW treatment for biohydrogen production (TSMB = two stage microbial treatment)

The initial phase extracts nutrients, wherein essential components are drawn from SWW and utilized for microalgal growth, which is then dewatered to capture the microalgal biomass that forms the foundation for subsequent hydrogen generation. Following this, the dewatered effluent is subjected to thermo acid pretreatment, a crucial procedure that disassembles the complex structures of biomass, hence improving the availability of carbohydrates, proteins, and lipids for microbial fermentation. This pretreatment enhances the digestibility of biomass for subsequent fermentation [25, 26].

After pretreatment, this stream undergoes dark fermentation, when bacteria convert organic substrates into biohydrogen and other volatile fatty acids without light.

During fermentation, the process pH is controlled manually or automatically. An automated system in the automatic pH control circuit regulates pH levels to support optimal fermentation, potentially improving efficiency and consistency. In manual pH control, pH is regulated manually, which may be beneficial for experimental comparison or in the absence of automatic control.

2.1 Microalgae

A carbohydrate-rich microalgae species called *C. vulgaris* ESP 6 was taken from fresh water and grown in a standard glass photobioreactor. The photobioreactor had a 500 mL working capacity and was filled with BG-11 media-containing cells that were first taken from agar and then cultured in liquid. The ambient temperature within the photobioreactor was maintained at 25 °C by a steady 200 rpm agitation, accompanied by 0.2 vvm aeration of 2% CO_2. Furthermore, continuous external light of 140 µmol/(m^2 s) was utilized to promote optimal development. Table 1 indicates the photobioreactor (1 L) environment providing the microalgae with the essential nutrients from the BG-11 culture media necessary to grow inside its limited space. These well-known pre-culture methods successfully got the microalgae strains ready for more research in the photobioreactor setting.

Table 1 BG-11 culture medium (200 mL)

Component	Mass	Component	Mass
$NaNO_3$	1.5 g	K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g	$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g	Na_2CO_3	0.02 g
$FeSO_4 \cdot 7H_2O$	0.006 g	EDTA	0.001 g
H_3BO_3	2.86 g	$ZnSO_4 \cdot 7H_2O$	0.222 g
$MnCl_2 \cdot 4H_2O$	1.81 g	$Na_2MoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g	$Co(NO_3)_2 \cdot 6H_2O$	0.0494 g

2.2 Slaughterhouse wastewater

SWW sourced from a nearby slaughterhouse first underwent a solid-liquid separation procedure that removed larger particles. Subsequently, it was filtered through a 140-micron sieve to remove finer solids. The effluent was preserved at temperatures below freezing for future utilization. Testing revealed that the wastewater had substantial concentrations of SS (1010–1100 mg/L), TS (1200–1410 mg/L), TOC (88–265 mg C/L), COD (450–680 mg/L), TN (90–110 mg/L), and TP (10–18 mg/L) and pH (8.0–8.5). To reduce the costs associated with water treatment, no comprehensive preparatory treatments, such as wastewater sterilization, were conducted prior to its subsequent processing with microalgae [27].

2.3 Treatment of slaughterhouse wastewater

It was found that the system with both bacteria and microalgae worked better at treating wastewater overall, especially at reducing COD, in which *C. vulgaris* ESP 6 is not very effective [28, 29]. Indigenous bacteria in SWW initiated the preliminary phase by employing the pretreatment method to reduce COD. To improve COD removal efficiency, several dilution ratios, from 25% to 100% with BG-11 medium (Table 1), were utilized for 40 days to optimize culture conditions in the SWW. The bacterial cultivation system was evaluated at 25 °C, with a capacity of 1 L, agitation at 150 rpm, and aeration at 0.2 mL/min using ordinary air.

The thermally treated wastewater, a product of the bacterial pretreatment, was then utilized for further microalgal culture, without the need for sterilization, at an inoculation concentration of 0.1 g/L. Regular sampling was performed to evaluate biomass output, carbohydrate content, and concentrations of COD, TN, and TP. The microalgal biomass cultured in the wastewater was harvested using centrifugation at 10 000 rpm for 5 min and freeze-dried. This biomass can be used as a feedstock in biohydrogen production via dark fermentation, demonstrating the practical application of this research in sustainable energy production [30, 31].

2.4 Thermal-acid pretreatment of microalgal biomass

Reducing sugars in microalgal biomass from *C. vulgaris* ESP 6 were released after a thermal-acid pretreatment utilizing a 1.5% HCl solution. In the experiment, 8 g of freeze-dried microalgae powder were put in a flask with 150 mL of the acid solution. The flask was placed under high pressure by autoclaving at 121 °C for 20 min. After acid hydrolysis, the pH of the resultant liquid hydrolysate was adjusted to 7 by adding 5 M sodium hydroxide. After that, salt crystals were taken out of the microalgae hydrolysate that had been neutralized by centrifuging it at 10 000 rpm for 5 min. The analysis of the obtained hydrolysate revealed a glucose-to-xylose ratio of around 5:1.

2.5 Biohydrogen production via dark fermentation

The anaerobic sludge effluent-isolated hydrogen-producing bacterial strain used in this study is *C. butyricum* CGS5. The strain has been deposited under accession number AY540109 in the NCBI nucleotide sequence database. Dark fermentation of the strain was performed in a 250 mL serum bottle with an operating volume of 150 mL, containing Endo medium (Table 2) and an initial inoculum size of 5%. Then, the system was incubated at 37 °C using an agitation speed of 150 rpm. The pH of the initial medium was adjusted using HCl to 7.5. Also, anaerobic conditions were maintained by flushing the serum bottle with argon gas and sealing it with an aluminum seal and rubber septum.

This study examined the feasibility of microalgae as a sustainable feedstock for biohydrogen production using microalgal biomass hydrolysate cultivated in two distinct mediums as a sucrose alternative in fermentations utilizing *C. butyricum*. Dark fermentation frequently leads to the buildup of acidic byproducts that lower the pH of the culture medium, hence limiting hydrogen generation [32–34]. To resolve this, pH was regulated at 5.5 via sodium hydroxide, supplied manually or via an automated control system. Manual pH changes were conducted in sealed vials under circumstances identical to previous tests. Conversely, a computerized regulation system employed a glass container with a pH electrode and continuous monitoring apparatus. Careful monitoring of pH levels via various management systems is intended to maintain optimal conditions for hydrogen production from algal biomass.

Table 2 Composition of the Endo medium

Component	Mass	Component	Mass
Sucrose	17.81 g/L	$NaHCO_3$	6.72 g/L
NH_4HCO_3	5.24 g/L	K_2HPO_4	0.125 g/L
$MgCl_26H_2O$	0.10 g/L	$MnSO_4 \cdot 6H_2O$	0.012 g/L
$FeSO_4 \cdot 7H_2O$	0.025 g/L	resazurin	0.001 g/L
$CuSO_4 \cdot 5H_2O$	0.005 g/L	L-cysteine-HCl	0.50 g/L
yeast extract	1.0 g/L	$CoCl_2 \cdot 6H_2O$	0.000135 g/L
$C_2H_3NaO_2S$	0.50 g/L		

2.6 Analysis

2.6.1 Gaseous and soluble products

Gas chromatography with a very sensitive thermal conductivity detector (100 ppm) was used to measure closely the gases produced when SWW was broken down without oxygen. A high-performance liquid chromatography system with a refractive index detector was used to precisely measure the different fermentation products made when organic matter broke down [35].

The primary liquid byproducts included acetic acid, butyric acid, the disaccharide sucrose, the monosaccharide glucose, and the pentose sugar xylose. This liquid chromatography system could measure the full range of organic acids and sugars produced. It was an important analytical tool for monitoring and evaluating the metabolic process's efficiency and biohydrogen production.

2.6.2 COD, TN, and TP concentrations

The COD in the SWW was assessed using established methods from the American Public Health Association (APHA) standard methods for the examination of water and wastewater [36]. The colorimetric Nash method was employed to ascertain total nitrogen levels. Simultaneously, the molybdenum-antimony anti-spectrophotometric method was employed to analyze the total phosphorus content. Precise measurement of COD, TN, and TP concentrations is essential for evaluating the effectiveness of remediation processes and ensuring that effluents comply with environmental discharge requirements [37, 38].

3 Results and discussion

3.1 Nutrient removal

Fig. 2 illustrates that removing phosphorus and COD exhibits comparable effectiveness across all concentrations, particularly at lower levels. The 25% concentration demonstrates optimal overall performance, exhibiting phosphorus and COD consumption reductions.

As the concentration of wastewater increases to 50%, 75%, and 100%, the efficiency in the removal of phosphorus and COD remains effective; nevertheless, the reduction rate somewhat declines due to the increased pollutant load. Conversely, total nitrogen removal is less effective at all doses, exhibiting high residual nitrogen levels after 7 days (Tables 3 and 4), particularly at the 75% and 100% concentrations doses, exhibiting elevated residual nitrogen levels after 7 days, particularly at the 75% and 100% concentrations.

This suggests that *C. vulgaris* is limited to absorbing or removing nitrogen from wastewater, particularly at excessive concentrations, where total nitrogen levels remain substantially high post-treatment. The lack of significant nitrogen removal highlights a challenge in employing

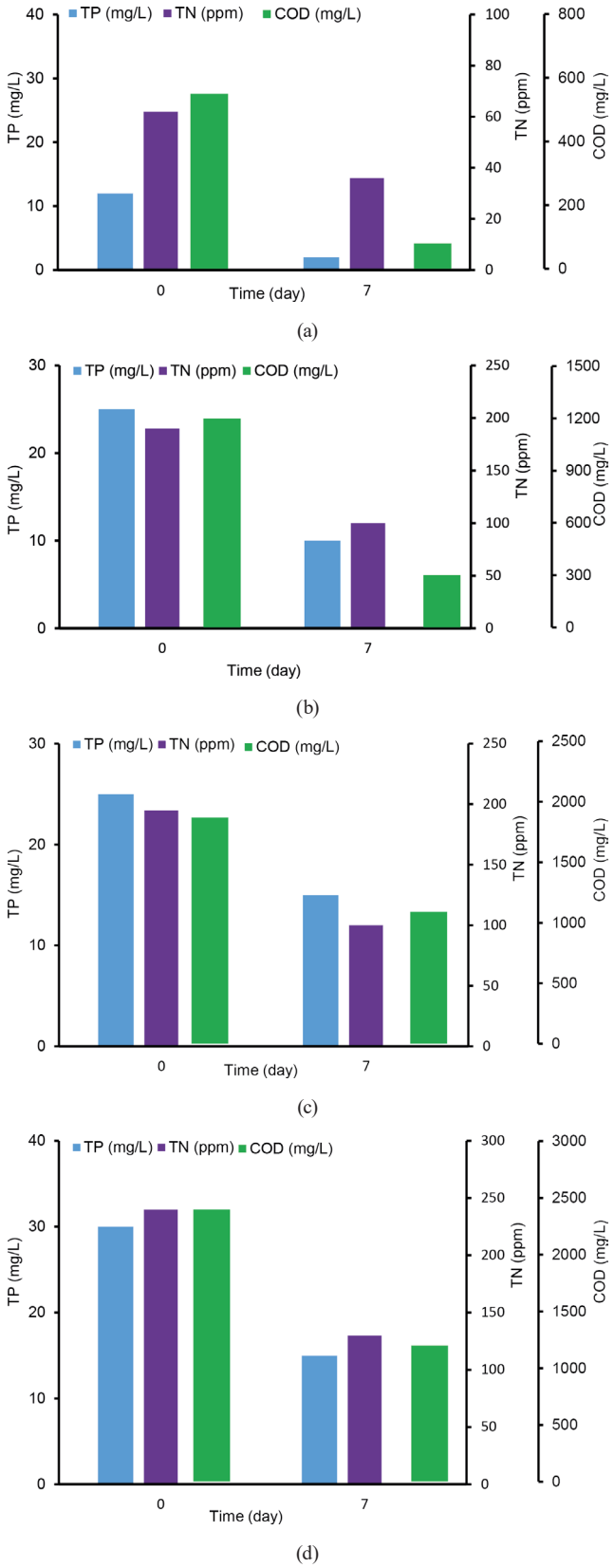


Fig. 2 *C. vulgaris* nutrient removal efficiency across different concentrations of SWW (a) 25%, (b) 50%, (c) 75%, (d) 100%

Table 3 Nutrient removal carbohydrate accumulation by *C. vulgaris* at different concentration of SWW after cultivation for 7 days

SWW (v/v)	COD Removal (%)	TP Removal (%)	TN Removal (%)	Glucose Content (%)
25%	41.5	81.0	83.0	43.0
50%	43.0	79.5	84.0	10.5
75%	44.5	41.5	45.4	3.5
100%	27.0	28.5	20.3	4.5

Table 4 Nutrient removal and carbohydrate accumulation by *C. vulgaris* at different concentration of SWW at different concentration of SWW after cultivation for 10 days

SWW Concentrations (v/v)	COD Removal (%)	TP Removal (%)	TN Removal (%)	Glucose Content (%)
25%	31.7	79.4	84.1	38.2
50%	36.3	82.3	96	42.2
75%	36.3	82.3	96	42.2
100%	36.3	82.3	96	42.2

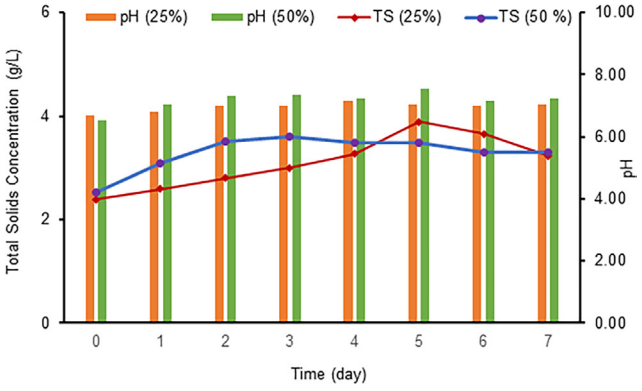
this organic treatment method in high-strength nitrogen wastewater environments [39, 40]. The results indicate that nutrient removal efficiency diminishes as the concentration of slaughterhouse wastewater rises.

The 25% concentration is the most effective for reducing phosphorus and COD. Total nitrogen removal is insufficient at all concentrations, with residual nitrogen persisting in the system even after 7 days. Elevated wastewater concentrations (75% and 100%) intensify this trend, exhibiting diminished phosphorus and COD reduction effectiveness alongside severely limited nitrogen removal.

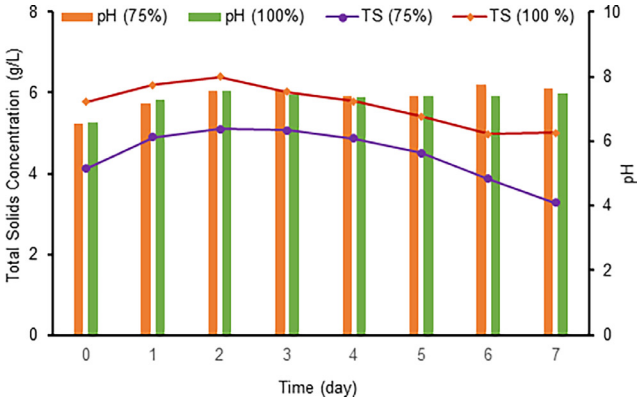
The data reveal optimal wastewater concentrations for enhancing nutrient removal efficiency with *C. vulgaris* ESP 6, especially for phosphate and COD; nevertheless, further treatment may improve nitrogen extraction.

3.2 Biomass production and pH

Fig. 3 shows how the biomass (shown as total solids concentration) and pH changed over 7 days for *C. vulgaris* grown in SWW at four different levels of dilution: 25%, 50%, 75%, and 100%. The 25% solution promotes moderated biomass growth, reaching its peak on the fifth day with a 50% increase, indicating consistent growth despite limited resources. At 50% dilution, the algae exhibit a 33.3% biomass increase, demonstrating that algae exhibit a 33.3% biomass increase, demonstrating consistent, stable growth without decline after the fifth day, indicating adequate nutrient availability for sustained development.



(a)



(b)

Fig. 3 *C. vulgaris* biomass growth and pH variation across different concentrations of SWW (a) 25% and 50% (b) 75% and 100%

The 75% dilution exhibits a 22.2% increase in the opening three days, followed by a decelerated growth rate and a final 11.1% increase, suggesting that nutrient depletion or environmental stress may restrict prolonged growth. At 100% dilution, rapid 30% growth occurs by the third day, followed by a significant fall, resulting in a 10% increase, indicating that excessive nutrient concentrations might initially stimulate growth but are ultimately detrimental to sustained cultivation.

The pH at a 25% dilution remains stable with a mere 7.7% increase, signifying a well-balanced environment that supports consistent biomass development as *C. vulgaris* flourishes within the favorable range of 6.5 to 7.0. Similarly, at 50% dilution, the 7.7% pH gradient reflects a steady environment, allowing uninterrupted growth without pH-related stress.

The 15.4% increase in pH after 75% dilution exhibits increased variability, which may clarify the rapid initial expansion followed by a slowdown as the system becomes less stable. At 100% dilution, the 15.4% pH increase indicates increased volatility, with sudden spikes promoting

initial growth, although environmental stress constrains sustained production, similar to patterns found at 75% dilution.

Both 25% and 50% SWW concentrations effectively extract TN, each resulting in a 75% reduction throughout the experiment. However, extraction rates vary. In the 25% dilution, TN sharply decreases from 80 ppm to 20 ppm by the third day, stabilizing afterwards, signifying that nitrogen removal is swift and thorough by that time. Conversely, in the 50% dilution, the reduction in TN is more gradual, commencing at 60 ppm and progressively decreasing to 15 ppm by the fifth day. The consistent fall in TN at the more significant dilution suggests that *C. vulgaris* continues to assimilate nitrogen during the entire duration, although at a reduced rate compared to the 25% dilution.

In summary, although both dilutions achieve the same final reduction, the 25% dilution expels nitrogen more rapidly, while the 50% dilution facilitates a longer prolonged absorption. The research study evaluated the effectiveness of two microalgae concentrations in wastewater treatment. At a 25% concentration, total nitrogen decreased from 80 ppm to 20 ppm by the third day, remaining constant afterwards. The differences in COD reduction among concentrations were more significant. At 25%, COD decreased significantly from 600 mg/L to 200 mg/L reduction. Although COD increased little in the following days, rising to approximately 300 by the fifth day, this showed a significant decrease in organic load.

Conversely, beginning at 800 mg/L with a 50% concentration, COD decreased to 350 on the third day, reflecting a decrease of 63%. Subsequently, COD surged, approaching the original level of 700 by the fifth day.

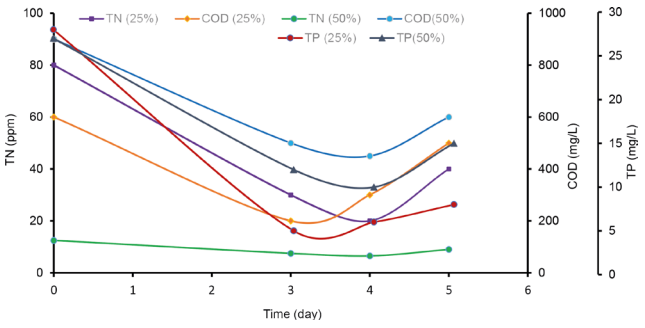
The final COD levels showed that while algae may initially decompose organics efficiently at high densities, the 50% concentration led to significantly greater organic byproduct or cellular waste production. The 25% concentration yielded more stable COD control with reduced volatility after the initial decline, rendering it the superior long-term solution for decreasing organic loads.

The elimination of total phosphorus exhibited a comparable trajectory at both concentrations, achieving an 80% reduction by the third day in each scenario. Phosphorus decreased from 25 mg/L to 5 by the third day, after that increasing to 10 by the fifth day, representing a 25% reduction. Phosphorus decreased to 5 mg/L by the third day, a reduction of 50%, but increased significantly to 15 mg/L by the fifth day. The increased phosphorus levels at the end of the experiment for the 50% concentration indicated a more significant release of phosphorus over time, likely attributable to biomass turnover or nutrient recycling within the system.

Consequently, although both concentrations initially excelled in phosphorus removal, the 25% concentration sustained more stable phosphorus levels, whereas the 50% concentration exhibited more significant fluctuations and less effective long-term phosphorus management.

3.3 Biomass growth

At a 25% concentration, the TS exhibit modest variations of approximately 10–15% (Fig. 4) during the experiment, signifying consistent and regulated biomass growth. The continual development of biomass indicates that *C. vulgaris* efficiently utilizes the available nutrients at this concentration, and the system's nutrient load is effectively regulated. This stability facilitates continuous growth without significant interruptions, demonstrating the system's capacity to preserve a balanced environment for efficient nutrient absorption. Conversely, at a 50% concentration, irregular changes in TS between 25–40% indicate variable variations in biomass accumulation. The increased nutrient concentration facilitates accelerated biomass development at specific intervals; however, subsequent declines suggest



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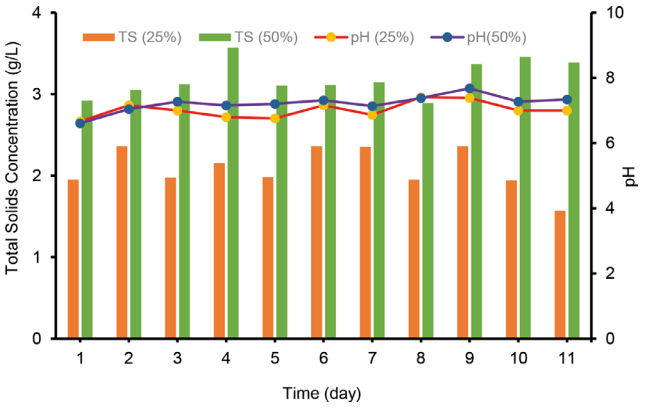


(b)

Fig. 4 *C. vulgaris* biomass growth and pH variation for 25%, and 50% concentrations of SWW (a) TN, COD and TP (b) pH and TS

that nutrient availability may fluctuate unpredictably due to nutrient depletion or the system's incapacity to consistently manage the increased nutritional load.

The significant fluctuations in TS illustrate that although the 50% concentration facilitates rapid development, it concurrently introduces increased instability relative to the 25% concentration, resulting in decreased stability in biomass production over time.

At a 25% concentration, the pH varies by approximately 0.8, ranging from 6.8 to 7.5, indicating moderate fluctuation and a well-buffered system favorable for consistent biomass growth. This stability fosters a balanced environment devoid of significant pH-related stress. At a 50% concentration, the pH varies by around 0.8 units, spanning from 7.0 to 7.8, exhibiting more significant fluctuation than the 25% concentration. This is still within the optimal range for *C. vulgaris* [41, 42].

3.4 Indigenous SWW bacteria

3.4.1 COD removal efficiency

At an initial concentration of 25% as shown in Fig. 5, the bacteria attain a COD removal efficiency of approximately 65%. This illustrates that despite lower concentrations, the bacteria effectively reduce COD levels, while the efficiency is relatively modest compared to higher concentrations. As the wastewater concentration increases to 50%, the COD removal efficiency rises to 85%, representing the highest efficiency observed. This significant enhancement indicates that the bacteria function optimally at moderate concentrations of organic matter, where the conditions are most conducive to COD degradation.

In 75% and 100% wastewater concentrations, the bacteria exhibit a high, although slightly reduced, efficiency of approximately 80%. This minor decrease from the peak efficiency at 50% suggests that although the bacteria remain effective at higher concentrations, they may have reached a threshold in their ability to remove COD efficiently beyond a particular range. As observed from Fig. 6, the bacteria exhibit their highest COD removal efficiency at 50%, showing a significant enhancement compared to the lower concentration of 25%. As concentrations are above 50%, efficiency slightly decreases and stabilizes, indicating that the bacteria achieve optimal performance at moderate concentrations, beyond which their COD removal capacity peaks.

This underscores the microorganisms' significant potential for effectively treating medium to high concentrations

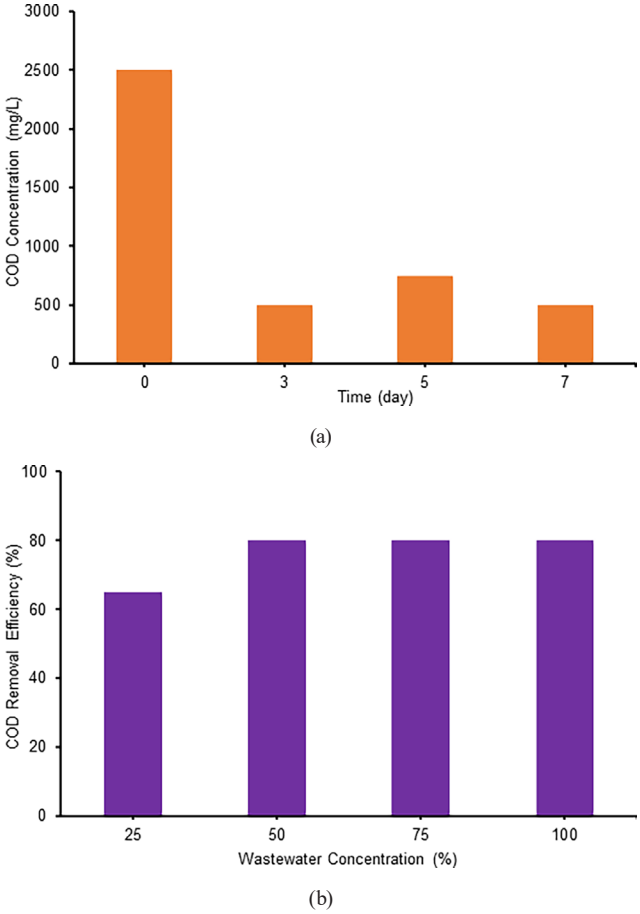
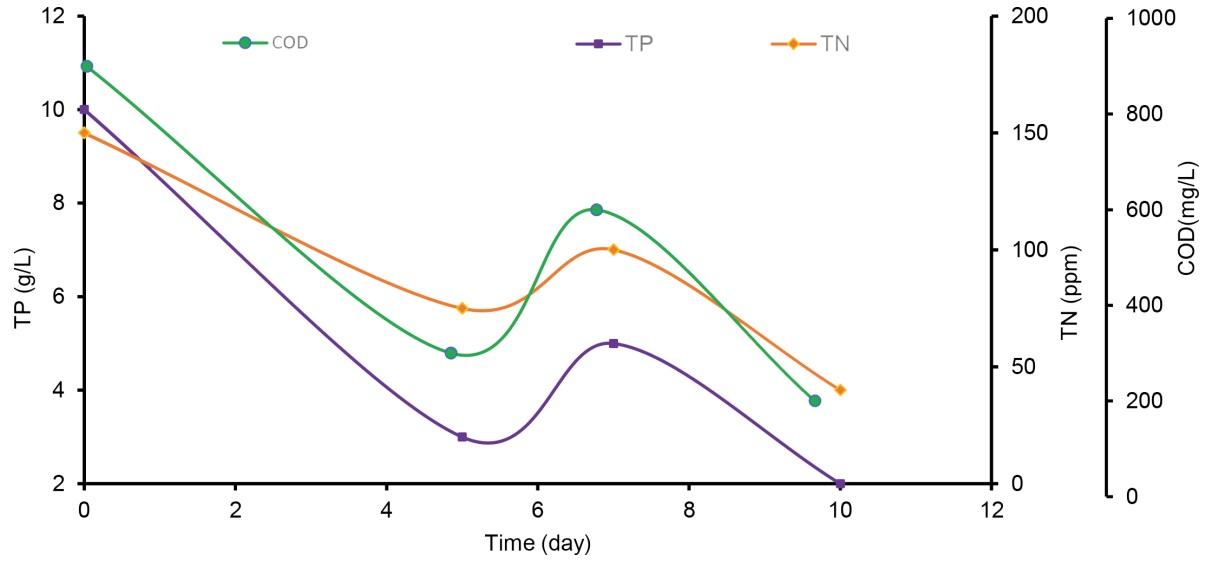


Fig. 5 (a) COD removal over a 7-day time frame for 100% SWW and (b) COD removal performance of indigenous WW bacteria for SWW of different concentrations

of slaughterhouse wastewater. The local wastewater bacteria exhibit rapid and effective COD removal from slaughterhouse wastewater in the initial treatment phase. During the first three days, there is an 80% decrease in COD concentration, falling from approximately 2500 mg/L to 500 mg/L, demonstrating the bacteria's exceptional capability in decomposing organic matter early.

Still, from day 3 to day 5, the COD assimilation slightly rises to 750 mg/L, which shows that the bacteria are not working as well, possibly because they are running out of compounds that break down easily. By day 7, the COD concentration decreases to around 600 mg/L, resulting in a total COD reduction of 76% for the whole treatment period.

Overall, bacteria are significantly influential in the initial stages of COD removal, with a decreasing impact as treatment progresses, likely due to increasingly resistant organic matter. The overall performance demonstrates a significant reduction in COD, with the bacteria maintaining approximately 76% efficiency by the end of the 7 days.



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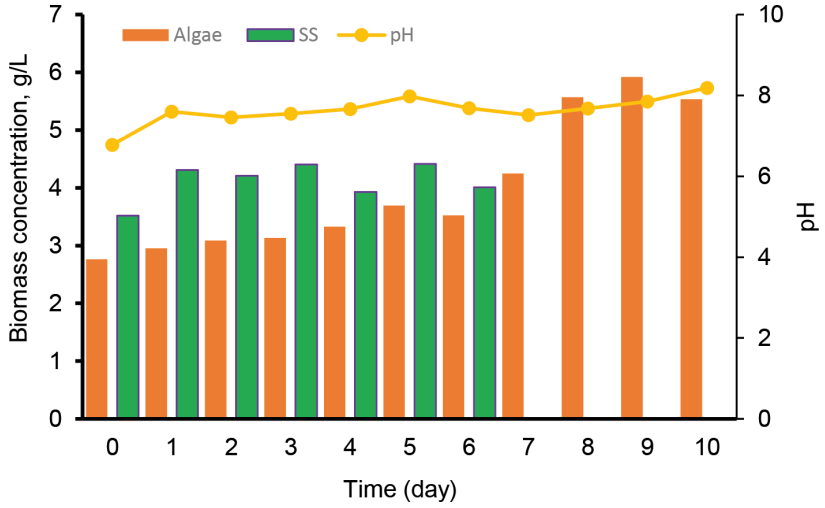


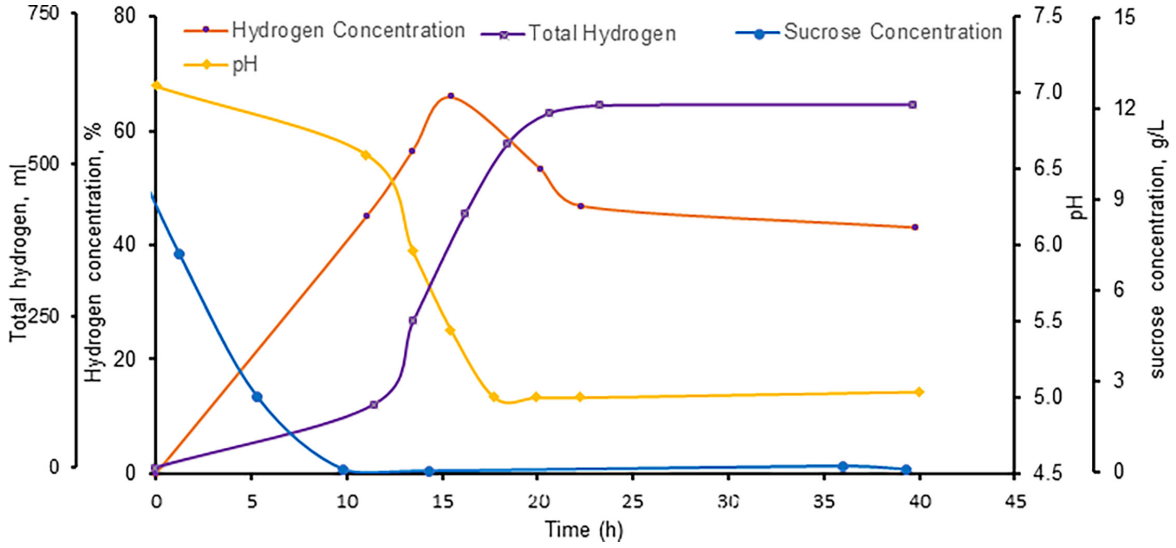
(b)

Fig. 6 Nutrient removal performance two-stage algae-bacteria culture system for *C. vulgaris* 100% SWW (a) COD, TN, TP and (b) algae, SS and pH

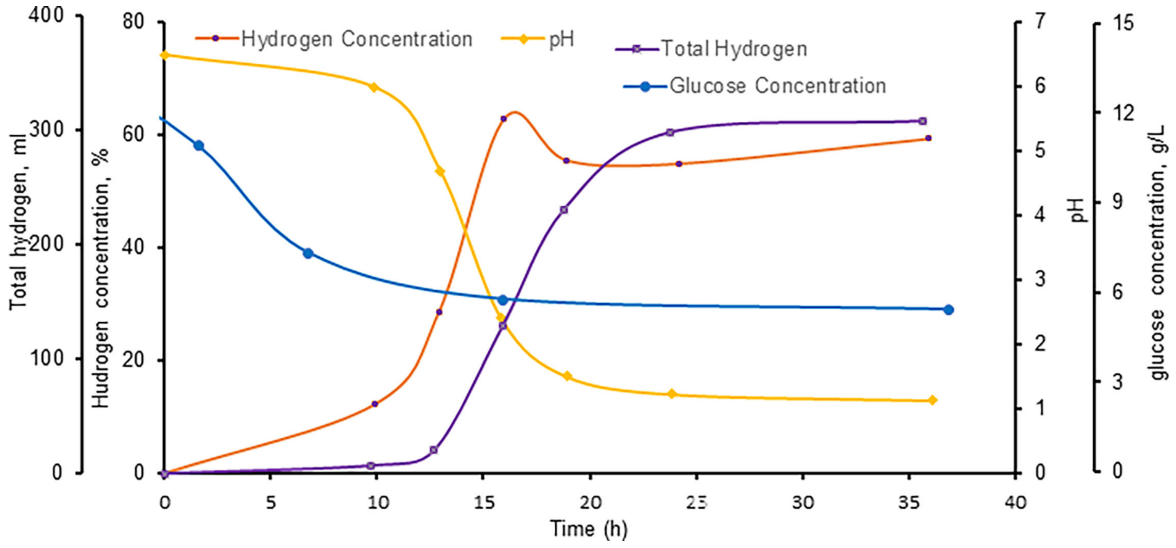
3.5 Two-stage algae-bacteria culture system

The analysis as seen in Fig. 7, reveals a significant decrease in COD, from approximately 1000 mg/L on the first day to about 200 mg/L by the end of day 10. A sharp decline in COD occurred over the first four days, followed by a minor increase around day 6, before resuming a steady decline. Total phosphorus begins at 10 g/L and decreases significantly to approximately 3 g/L by day 5. Following a slight increase between days 5 and 7, it decreases again, reaching approximately 0 g/L by day 10. This demonstrates that the algae absorbed a significant amount of phosphorus, which they almost eliminated by the end of the treatment. Total nitrogen consistently decreases during treatment, starting at 150 ppm and decreasing to approximately 25 ppm by day 10. This indicates ongoing nitrogen removal by bacteria via processes like nitrification and denitrification.

During the early phase (day 0 to day 4), there was a significant decrease in all three parameters—COD, TP, and TN—signifying a highly productive period for algae and bacteria. The algae efficiently eliminate TP, while bacteria decompose COD and reduce TN. The two organisms operated efficiently in concert throughout this phase. In the mid-phase (day 5 to day 7) period, a little increase in COD and TP was seen, likely attributable to cellular degradation from algae or bacteria, which released organic matter and nutrients into the system. Nevertheless, TN continued to fall gradually. After this brief fluctuation, the system achieved stability, and the elimination of all contaminants recommenced. During the last phase (day 7 to day 10), TP had a significant decrease, approaching negligible levels, while COD further decreased to approximately 200 mg/L, indicating the system's regained performance. Total



(a)



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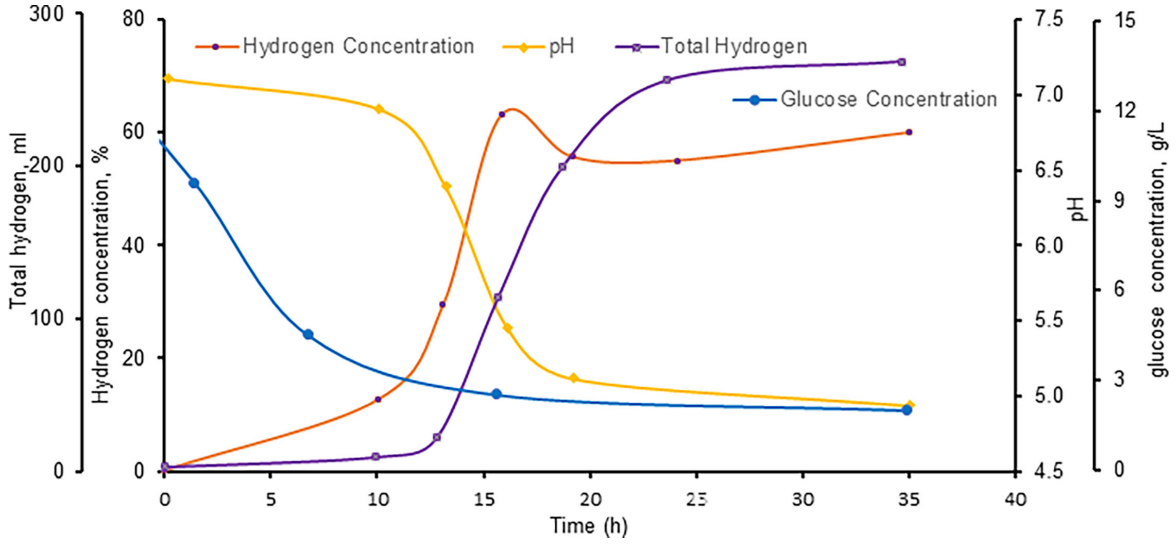


(c)

Fig. 7 Biohydrogen production by *C. butyricum* CGS5 using (a) sucrose, (b) hydrolysate of microalgae cultured in BG-11, and (c) hydrolysate of microalgae cultured in SWW as the carbon source

nitrogen levels also continued to decline, indicating effective nitrogen removal. This phase means the algae-bacteria growth system attains optimal efficiency after the treatment cycle, successfully diminishing all contaminants.

3.6 Biohydrogen production

3.6.1 Sucrose as the carbon source

During the first phase, the concentration of hydrogen gradually rose, which meant that hydrogen production began as *C. butyricum* broke down sucrose in its growth phase. Approximately 15 h in, the hydrogen concentration peaked at nearly 80%, indicating this interval represented the most excellent output, as a substantial quantity of Sucrose had been depleted. After 15 h, the hydrogen concentration decreased gradually, suggesting a deceleration of the fermentation process as the available sucrose decreased and hydrogen production decreased.

Around 10 h, the sucrose concentration began at roughly 20 g/L and steadily reduced, indicating that *C. butyricum* effectively metabolized Sucrose to produce hydrogen and other byproducts.

After around 20 h, the sucrose was depleted mainly, confirming its role as the primary substrate facilitating hydrogen generation. The reduction of sucrose corresponded with the peak and subsequent decrease in hydrogen concentration, as hydrogen production diminished once the sucrose was consumed. Initially, the pH remained relatively steady at approximately 6.5; however, as hydrogen generation intensified between 10 and 20 h, the pH suddenly dropped to roughly 4.8. The reduction in pH was likely due to the accumulation of acidic byproducts, including volatile fatty acids, during fermentation. After 20 h, as sucrose was nearly consumed and hydrogen production reduced, the pH slightly improved and steadied at approximately 5, signifying the end of active fermentation and acidogenesis.

From 0 to approximately 10 h in the lag phase, overall hydrogen generation was insignificant, indicating a substantial lag in hydrogen accumulation. From 10 to 25 h, total hydrogen generation increased significantly, following the trend of hydrogen concentration, indicative of the phase during which sucrose was actively fermented. After 25 h, overall hydrogen production stabilized, indicating that the fermentation process was approaching the end, as most sucrose had been utilized, and hydrogen generation had decreased. When *C. butyricum* broke down sucrose, the hydrogen concentration went up. This proved that sucrose is a good carbon source for making biohydrogen. The consistent decrease in sucrose concentration was directly correlated with increased hydrogen production, peaking when sucrose was efficiently utilized. The reduction in pH coincided with the peak of fermentation activity occurring between 10 and 20 h, during which hydrogen production reached its highest point. Throughout this interval, the accumulation of acidic fermentation byproducts resulted in a significant decrease in pH. Upon the depletion of sucrose and the reduction of hydrogen production, the pH stabilized, signifying the end of the most vigorous phase of fermentation.

As sucrose was nearly consumed around the 20-hour mark, the hydrogen content decreased, indicating that *C. butyricum* had predominantly consumed its energy supply for hydrogen production. Subsequently, the overall hydrogen production curve steadied, indicating the end of active hydrogen formation as the fermentation process was completed.

3.6.2 Hydrolysate of microalgae cultured in BG-11

The fermentation process utilizing *C. butyricum* for hydrogen production exhibits unique variations over time. During the initial period of up to 10 h, the concentration of hydrogen is basically insignificant and progressively increases as *C. butyricum* begins to metabolize the available glucose. Glucose concentration initiates at around 12 g/L and gradually decreases as it is utilized for hydrogen production. During this phase, the pH remains constant at approximately 6.5, indicating that microbial activity is still relatively low.

During the active fermentation phase, lasting between 10 and 15 h, hydrogen concentration reaches approximately 80%, signifying the maximum glucose consumption rate. Glucose levels continue to decline sharply throughout this period, indicating that glucose is efficiently utilized as a carbon source. Concurrently, the pH decreases markedly to approximately 5, presumably due to the accumulation of acidic byproducts from rapid glucose metabolism.

After 15 h, hydrogen concentration begins to decrease, indicating glucose depletion. After 20 h, glucose is nearly exhausted, coinciding with the reduction in hydrogen production. As fermentation drops, the pH stabilizes and gradually returns to approximately 6, indicating the end of active fermentation and an absence of acidic byproduct production.

After 20 h, total hydrogen production stabilizes, signifying that the fermentation process is approaching completion. Most glucose has been utilized, resulting

in a slowdown or complete halt of hydrogen production. This peak represents the conclusion of active fermentation, indicating that the glucose supply directly influences hydrogen production.

During fermentation, a significant correlation exists between glucose consumption and hydrogen production. As glucose is digested, hydrogen production increases, with the maximum hydrogen concentration aligning with the peak glucose consumption. The decrease in glucose concentration immediately affects the rate of hydrogen production, highlighting the vital role of glucose as a carbon source for biohydrogen synthesis.

3.6.3 Hydrolysate of microalgae cultured in SWW

The study of hydrogen production shows a clear link between the amount of glucose used and the amount of hydrogen produced during the *C. butyricum* fermentation process. Hydrogen levels were initially low but increased significantly during the first 10–15 h, accounting for 10–15% of the total fermentation time. This phase saw moderate hydrogen production as glucose was metabolized. By the 15-hour mark, hydrogen production peaked at 80%, coinciding with vigorous glucose consumption, representing about 60% of the fermentation duration.

The starting glucose concentration of approximately 12 g/L decreased steadily, reaching near zero by the 20-hour mark, indicating a direct relationship between glucose availability and hydrogen production. The pH remained stable around 6.5 for the first 10 h but dropped sharply to around 5 during the peak production phase (10–15 h) due to the accumulation of acidic byproducts. After this peak, as glucose consumption slowed, pH values stabilized between 5 and 5.5.

Overall, the most significant hydrogen output occurred between 10 to 20 h, representing 50% of the fermentation time, while production plateaued after 25 h as glucose was nearly fully consumed. The data suggests that glucose was the primary carbon source driving hydrogen production, with a notable drop in pH during peak output, indicating the buildup of acids. The process concluded around 25 h, marking the end of significant hydrogen liberation due to substrate exhaustion.

3.7 The pH control strategy

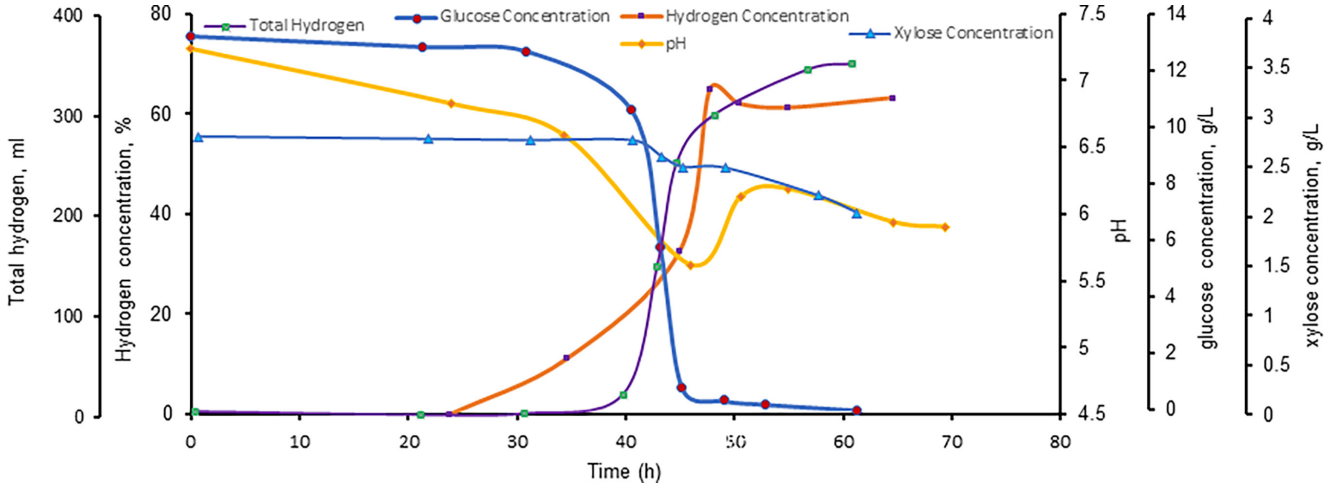
3.7.1 Manual

As hydrogen production began, the anaerobic bacteria *C. butyricum* gradually metabolized glucose and xylose from enzymatically treated microalgae, favoring glucose as observed in Fig. 8. As this principal sugar source reduced, hydrogen levels shot up. Peak output occurred between 20 and 40 h when hydrogen accumulation peaked, mostly from glucose breakdown. As glucose levels dropped at this peak phase, xylose use increased, indicating a substrate preference shift. The delay in xylose consumption suggested glucose was digested first. Hydrogen generation decreased as both fuels became scarcer, reducing microbial activity.

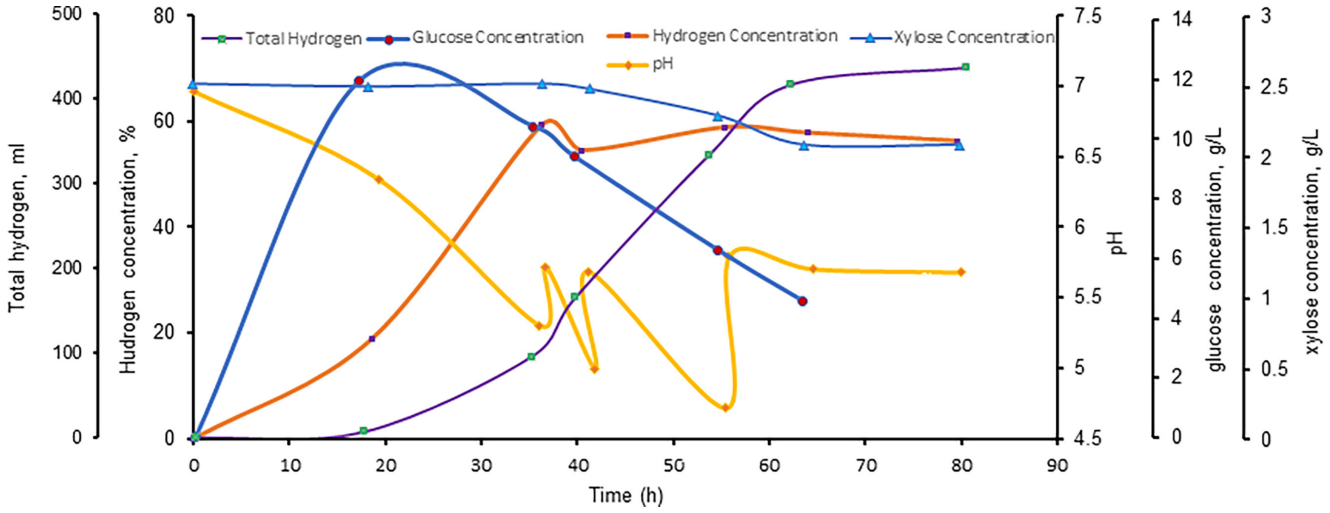
Early in the process, the pH was steady between 6.5 and 7.0. However, when metabolism increased, and maximal hydrogen levels approached, acid byproducts such as organic acids accumulated, lowering pH dramatically between 10 and 40 h. This pH reduction coincided with peak production. After the peak at 40 h, manual control kept pH between 5.5 and 6.0 to avoid over-acidification while allowing the process to continue at a reduced rate when substrates became scarce.

The glucose concentration started at 12 g/L and progressively decreased as it was consumed to make hydrogen, approaching zero by 40 h. This depletion coincided with maximal hydrogen production, suggesting glucose drove early fermentation. From 2.5 g/L, xylose usage gradually fell, trailing glucose. This revealed that *C. butyricum* switched to xylose when glucose was primarily used. Xylose consumption continued until 60 h, prolonging hydrogen synthesis and microbial exercise after glucose ran out. The early lag saw little hydrogen generation as microbial cells adapted to their new environment and broke down glucose and xylose. Total hydrogen production increased significantly between 10 and 40 h during the active fermentation phase when glucose and xylose were broken down well. Since accessible substrates were essentially depleted after 40 h, hydrogen generation plateaued, indicating fermentation had slowed.

The plateau showed that carbon resources were the only source of hydrogen production after that point, yielding l hydrogen. Hydrogen generation and glucose/xylose intake were linked. Hydrogen production initially increased due to fast glucose metabolism. As glucose dwindled, xylose intake increased, supporting hydrogen generation until both components were practically gone. This successive use of glucose and xylose prolonged hydrogen generation during fermentation. The most active phase of fermentation, when acidic byproducts from glucose and xylose breakdown dropped pH sharply, occurred between 10 and 40 h. Avoiding over-acidification after 40 h with manual pH stabilization lengthened the fermentation process. As substrates decreased, hydrogen creation slowed.



(a)



(b)

Fig. 8 Biohydrogen production by *C. butyricum* CGS5 using hydrolysate of microalgae cultured in SWW as carbon source with (a) manual and (b) automatic pH control strategy

Hydrogen concentration dropped significantly between 40 and 60 h when glucose and xylose were nearly exhausted, ending active hydrogen synthesis. The overall hydrogen production curve stabilized, confirming that fermentation had slowed, and the technique was nearing completion with limited hydrogen creation.

3.7.2 Automatic pH

C. butyricum starts fermentation by turning the microalgae's glucose and xylose into hydrogen gas. In the first phase, glucose is the primary carbon source for metabolism, whereas xylose absorption is comparatively lower. During the ideal hydrogen production phase lasts 20–30 h, glucose levels drop sharply while xylose consumption rises. This shows that *C. butyricum* switches substrates as glucose levels drop, keeping microbial activity high. The pH stability is crucial to successful fermentation, maintained between 5.5 and 6.5 by an automated control system. This stability creates optimal circumstances for microbial action and prevents excessive acidity that might hinder hydrogen generation. The constant pH environment, which supports continuous reactions, enables the prolonged fermentation duration.

Glucose intake starts at around 12 g/L and gradually decreases as it aids in hydrogen production, nearly exhausting by 30 h. Xylose absorption starts gradually, escalating after 10 h and continuing until almost depleted at around 60 h. Xylose is an alternative carbon source following glucose depletion, sustaining hydrogen production beyond the first substrate exhaustion and prolonging the fermentation period. The lag phase generates minimal hydrogen as microbial cells develop and ready themselves for vigorous

fermentation. Hydrogen generation intensifies significantly within 10 to 30 h, coinciding with the peak phase, during which glucose and xylose are efficiently used. Following a peak at around 30 h, production stabilizes and declines when glucose and xylose are depleted.

Automated pH regulation maintains stability between 5.5 and 6.5, avoiding the typical sharp decrease caused by the buildup of acidic byproducts during fermentation. This stability guarantees excellent operations and facilitates continuous fermentation without obstructing the microorganisms responsible for hydrogen generation.

4 Conclusion

The study on nutrient removal and algal growth using *C. vulgaris* revealed that a 25% wastewater (WW) concentration was optimal for consistent nutrient reduction. The study on nutrient removal and algal growth using *C. vulgaris* revealed that a 25% wastewater (WW) concentration was optimal for consistent results. At this dilution, phosphorus and COD decreased by 80% and 66% within three days, with phosphorus removal remaining stable throughout. Conversely, the 50% concentration initially mirrored these reductions but displayed fluctuations, with COD nearly reverting to its original level by day five. Both 25% and 50% concentrations reduced total nitrogen by 75%, but the 25% concentration facilitated faster nitrogen extraction. Biomass generation was more consistent at 25%, increasing by 50% compared to 33.3% at the higher concentration, indicating variability due to nutrient availability. The pH remained stable at both concentrations, with the 25% solution being less prone to fluctuations, making it preferable for long-term wastewater management.

Indigenous SWW bacteria showed significant COD removal capabilities, achieving 65% efficiency at 25% concentration and improving to 85% at 50%. Even at higher concentrations (75% and 100%), the bacteria maintained an 80% contaminant removal rate, although a slight decline in efficiency was noted at midpoint concentrations. Initially, the bacteria reduced COD by 80% within three days, from 2500 mg/L to 500 mg/L, but their viability decreased over time, with total COD reduction reaching 76% by day seven. This highlights the bacteria's effectiveness in managing medium to high concentrations of slaughterhouse wastewater, particularly in the early treatment stages.

The treatment resulted in a significant decrease in COD, dropping from approximately 1000 mg/L to 200 mg/L, with a rapid decline in the first four days followed by a temporary increase. Total phosphorus decreased from 10 g/L to nearly zero by the experiment's end, indicating effective algae utilization of phosphorus. Total nitrogen levels also fell by almost 80%, from 150 ppm to 37.5 ppm, demonstrating active bacterial involvement in nitrogen extraction. The system exhibited exceptional performance, with algae and bacteria working synergistically to achieve near-complete pollutant elimination.

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Authors' contribution

Reena Vaidya carried out the experiments and analysis, Nitin Raut conceptualized and designed the methods, and Amal Al Saadi and Mostafa Ghasemi jointly analyzed the findings and wrote the manuscript draft. All the authors actively developed their analysis and refined their work for dissemination.

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