Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Outdoor biohydrogen production by thermotolerant Rhodopseudomonas pentothenatexigens KKU-SN1/1 in a cluster of ten bioreactors system

Netchanok Punriboon
Khon Kaen University
Jutaporn Sawaengkaew
Khon Kaen University
Polson Mahakhan
polson@kku.ac.th

Khon Kaen University

Research Article

Keywords: High-temperature, Outdoor production, Photo-fermentation, Rhodopseudomonas harwoodiae, Rhodopseudomonas pentothenatexigens, Scale-up

Posted Date: January 9th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3837038/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Bioprocess and Biosystems Engineering on March 15th, 2024. See the published version at https://doi.org/10.1007/s00449-024-02996-4.

Abstract

In tropical regions, the viability of outdoor photo-fermentative biohydrogen production faces challenges arising from elevated temperatures and varying light intensity. This research aimed to explore how high temperatures and outdoor environments impact both biohydrogen production and the growth of purple non-sulfur bacteria. The findings revealed the potential of *Rhodopseudomonas* spp. as a robust outdoor hydrogen-producing bacteria, demonstrating its capacity to thrive and generate biohydrogen even at 40°C and under fluctuating outdoor conditions. Notably, *Rhodopseudomonas pentothenatexigens* KKU-SN1/1 exhibited the highest cumulative biohydrogen production of 400 mL/L under outdoor conditions. In addition, the outdoor enhancement of biohydrogen production was achieved through the utilization of a cluster of ten bioreactors system. The outcomes demonstrated a notable improvement in biohydrogen production efficiency, marked the highest daily biohydrogen production was 493 mL/L/day. Significantly, the highest biohydrogen production rate was noted to be 17 times greater than that observed in conventional batch production methods. This study is the first report represents the inaugural utilization of *R. pentothenatexigens* for sustained biohydrogen production in the outdoor conditions, facilitated by the cluster of ten bioreactors system over an extended operational period.

Introduction

Currently, both demands for energy sources and greenhouse gas emissions are increasing due to the growth of industry [1]. The primary energy source is fossil fuels, which is regarded as non-renewable. The use of fossil fuels contributes to carbon dioxide emissions which are harmful to the environment, thereby contributing to global warming [2]. As a result, it is vital to explore possible renewable energy sources as an alternative to fossil fuel consumption. Hydrogen is an enticing alternative energy carrier that has a high energy content of about 142 kJ/g [3]. It is considered a form of clean energy because it releases only water when burned and consequently has a low impact on the environment, making it widely regarded as the most environmentally friendly fuel [4]. In addition, hydrogen energy has a wide range of possible applications, including the generation of heat, electricity, backup power, and vehicle power [5]. Therefore, hydrogen production has previously been considered; however, the production cost was high, especially by industrial methods [6]. Low-cost hydrogen production would, then, be of great interest.

The biological hydrogen production process is more environmentally friendly and utilizes less energy than other technologies of hydrogen production [7]. In addition, its moderate operating condition makes biological hydrogen production a cost-effective and environmentally benign option-offering great efficiency and the possibility to utilize renewable biomass sources [8]. Numerous bacteria can produce hydrogen by microbial enzymatic activity at the optimal pressure and temperature, with various processes according to microbe type [9]. Among the biological hydrogen production processes, the photo-fermentative hydrogen production of photosynthetic bacteria theoretically produces large amounts of hydrogen [10]. Therefore, biohydrogen production by purple non-sulfur bacteria (PNSB) has been of interest. PNSB are phototrophic microorganisms whose metabolism is unique in that it can grow in different modes [11], making them a popular choice for hydrogen production. Under anaerobic-light

conditions, PNSB can convert organic acid produced from the consumption of organic material into hydrogen and carbon dioxide [12]. The advantages of using PNSB for photo-fermentative hydrogen production include an anaerobic function that prevents oxygen from inhibiting nitrogenase activity, a broad spectrum of light, the ability to utilize a diverse range of raw materials, and a high substrate conversion efficiency [13].

Temperature and light are important factors for the biohydrogen production of PNSB. Temperature is important for metabolic processes and hydrogen production of photosynthetic bacteria. The optimum temperature for hydrogen production of PNSB is 30–36°C [14–17]. High temperatures may inhibit both enzyme activity and the proteins involved in bacterial cell growth and hydrogen production [18]. At temperatures of 33°C and 37°C, biohydrogen production and bacterial cell growth of wild-type strain and mutant strain of PNSB are reduced [19]. In addition, light is essential for the photo-fermentative hydrogen production of PNSB because the reaction, electron transport, ATP synthesis, and hydrogen production all require light energy [20]. However, the light source requires electricity, the main cost of photo-fermentative hydrogen production. Natural light is an attractive alternative to reduce the costs of photo-fermentative hydrogen production. However, the use of natural light sources under outdoor conditions may lead to problems with photo-fermentative biohydrogen production by PNSB, which is related to light and temperature, as follows: (I) the overexposed state during the midday hours reduces the efficiency of light energy conversion to biohydrogen production of PNSB, (II) the fluctuations of light intensity and temperature throughout the day and night negatively affects bacterial cell growth and the photo-fermentative hydrogen production of PNSB, and (III) the presence of rain and clouds and the cycle of day and night, resulting in a drop in light intensity and a change in temperature, negatively affects photo-fermentative hydrogen production, with little or no hydrogen production seen during the night, and bacterial activity only restored in the presence of light [21–23]. In addition, Thailand is close to the equator and has a tropical climate. The average ambient temperature is extremely high, and the hot season is quite lengthy. Therefore, the ability of microorganisms to tolerate high temperatures and adaptability during the day is particularly important when performing photo-fermentative hydrogen production under outdoor conditions. Some reports have demonstrated that certain PNSB can survive and produce hydrogen at high temperatures. *R. pentothenatexigens* and *R. harwoodiae* are mesophilic bacteria capable of growing in a wide temperature range; the optimum range temperatures for *R. pentothenatexigens* and *R. harwoodiae* growth are 25–50°C and 25–40°C, respectively [24, 25]. Previous research by our team reported that the biohydrogen production of *R. pentothenatexigens* KKU-SN1/1 at high temperatures (40°C) was greater than at ambient temperature (26–29°C) and was identified as a thermo-tolerant photosynthetic biohydrogen producing bacteria capable of producing hydrogen at high temperatures [26]. Biohydrogen production of *R. harwoodiae* NM3/1–2, 2M, 14M and KK(NM)3 – 2 at ambient temperature (27 ± 2°C) was higher than under outdoor conditions [27]. In addition, *R. pentothenatexigens* and *R. harwoodiae* have rarely been reported on for hydrogen production.

The aim of this study was to improve the capacity of biohydrogen production by PNSB under outdoor conditions using the cluster of ten bioreactors system (CTBS) for continuous productivity and a reduction

in production costs. The effect of high temperatures and outdoor conditions on the biohydrogen production and bacterial cell growth of *Rhodopseudomonas* spp. was also investigated.

Materials and methods

Microorganisms and culture conditions

Five strains of PNSB, *R. pentothenatexigens* KKU-SN1/1, *R. harwoodiae* NM3/1–2, *R. harwoodiae* 2M, *R. harwoodiae* 14M and *R. harwoodiae* KK(NM)3–21 isolated from environmental samples from areas in the northeast of Thailand [26, 27], were obtained from Hydrogen-Enzyme Laboratory, Department of Microbiology, Faculty of Science, Khon Kaen University, Thailand, and used in this research. PNSB were cultured in modified Ormerod's medium [26, 28], containing K_2HPO_4 (0.9 g/L), KH_2PO_4 (0.6 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), $CaCl_2{\cdot}2H_2O$ (0.075 g/L), $FeSO_4{\cdot}7H_2O$ (0.0118 g/L), EDTA•2Na (0.02 g/L), p-aminobenzoic acid (0.001 g/L), thiamine HCl (0.001 g/L), nicotinic acid (0.001 g/L), malic acid (7.6 g/L) as a carbon source and glutamic acid (11.0 g/L) as a nitrogen source. The initial pH of the medium was adjusted to 6.7 using 1M sodium hydroxide before autoclaving at 121°C for 20 min. Nitrogen gas was employed to create anaerobic conditions. The starer culture was incubated at ambient temperature (30–40°C) under an illumination of 10,000 Lux using warm white LED lamps.

Simple bioreactor

The simple bioreactor was made from polyethylene terephthalate (PET) bioreactor with a working volume of 300 mL (diameter = 55 mm, height = 150 mm) and 1,500 mL (diameter = 85 mm, height = 300 mm). The bottle was cleaned with 70% ethyl alcohol and dried before being used for biohydrogen production. The starter culture (10%, initial $OD_{660nm} \approx 0.4$) was inoculated in the sterilized simple bioreactor containing modified Ormerod's broth (90%) and sealed by a sterilized silicone rubber stopper puncture with a sterilized needle. The top of a sterilized needle was connected to the hose through a 0.45 μm pore filter. The other end of the hose was connected to a measuring cylinder to record the volume of gas production from the water replacement method [29].

Biohydrogen production at high temperature

PNSB were cultured in a 300 mL working volume simple bioreactor and incubated under an illumination of 10,000 Lux using warm white LED lamps. The simple bioreactor was placed in a clear glass water bath controlled at 40°C. The experiments were carried out for 20 days. Samples were collected every 24 h for investigation of bacterial cell growth, pH value during fermentation, and biohydrogen production.

Biohydrogen production under outdoor conditions

PNSB were cultured in 300 mL and 1,500 mL working volume of simple bioreactor. The cultures were incubated under natural light, without control over light intensity throughout the experiment. The simple bioreactor was situated inside a clear glass water bath characterized by dimensions of 300 mm in height,

1,200 mm in length, and 300 mm in width. The water within the bath was filled to a volume 80% of the height of bioreactor, serving the purpose of temperature regulation to prevent it from exceeding 40°C. The experiments were conducted from January to February under natural conditions in Khon Kaen, Thailand. The daytime ambient temperature was in a range of 21–40°C. The night-time ambient temperature was in a range of 21–30°C. The average light intensity in the daytime was approximately 89,820 Lux. The highest light intensity in the daytime was around 182,000 Lux at 1 p.m. The experiment was exposed to natural light daily from around 6 a.m.–6 p.m. Samples were collected every day at 4 p.m. to investigate bacterial cell growth, pH value during fermentation, and biohydrogen production for 20 days.

Biohydrogen production by CTBS

CTBS consists of several 1,500 mL simple bioreactors as in previously experiment which are separated from each other, a gas pipeline, a gas storage section, and a light source (**Fig. 1**). CTBS consists of 3 phases of operation: the start phase as the new starter cultures and new media are added by simple bioreactor replenishment every 2 days (**Fig. 1a**), causing overlapping biohydrogen production, the complete phase as the system completes 10 bioreactors (**Fig. 1b**), and the continuous phase such as the No. 1 simple bioreactor on day 20, where no hydrogen production is removed from the system, and a new simple bioreactor is added in its place (**Fig. 1c**), resulting in continuous exposure to hydrogen production.

Analytical methods

Bacterial cell growth was measured by spectrophotometer at the wavelength of 660 nm (Double beam UV/Vis spectrophotometer OPTIZEN 3220UV, Mecasys, Korea) and converted to dry cell weight with a standard curve constructed by harvesting bacterial cell and drying at 70°C until constant weight. The pH meter was used to measure the pH of liquid samples (Eutech pH 700, Eytech Instruments Pte Ltd, Singapore). The volume of gas production was observed from the replacement of gas accumulated in the measuring cylinder. Gas chromatography (model GC-2014, Shimazu Corp., Japan) was used to determine the purity of biohydrogen from the headspace of the simple bioreactor by using a thermal conductivity detector and a molecular sieve 5A column. The injector temperature was maintained at 180°C. The detector temperature was set to 180°C. The carrier gas was argon at a flow rate of 25 mL/min. The oven temperature was maintained at 130°C. The temperature was measured using a thermometer. A lux meter measured light intensity.

Statistical analysis

All experiments were replicated three times and error bars are included in the figures. Microsoft Excel was used to calculate mean and standard deviations. The IBM SPSS version 28.0.1.0 (142) was used for One-Way ANOVA analysis.

Results and discussions

Effect of high temperature on growth and biohydrogen production

PNSB were cultured in a 300 mL simple bioreactor in batch mode to examine the capability of biohydrogen production and bacterial cell growth at 40°C in anaerobic-light conditions. The results showed that biohydrogen production of PNSB began after 1 day of cultivation (Fig. 2). Biohydrogen production from KKU-SN1/1, KK(NM)3–21, and 14M were detected from day 1 to day 7 and ended on day 8. Biohydrogen production from NM3/1–2 and 2M were detected from day 1 to day 9 and ended on entering day 10. The highest cumulative biohydrogen production was 223 mL/L by NM3/1–2, followed by 2M, KK(NM)3–21, KKU-SN1/1 and 14M were 182 mL/L, 172 mL/L, 143 mL/L, and 132 mL/L, respectively. The highest maximum biohydrogen production rate was 1.81 mL/L/h from KKU-SN1/1 and KK(NM)3–21 on day 6 and day 3, respectively. The maximum biohydrogen production rate of NM3/1–2 was 1.67 mL/L/h on day 4. The maximum biohydrogen production rate of 14M was 1.39 mL/L/h on day 3. The maximum biohydrogen production rate of 2M was 1.34 mL/L/h on day 5.

Bacterial cell growth of PNSB showed similar trends in Fig. 3. From day 0 to day 3, bacterial cell growth increased dramatically. After day 3, cell concentration gradually increased. From day 9 to day 20, cell concentrations increased slightly. At the end of photo-fermentation, KKU-SN1/1 had the highest average dry cell weight of 3.07 g/L, followed by 2M, 14M, NM3/1–2 and KK(NM)3–21 had an average dry cell weight of 2.65 g/L, 2.54 g/L, 2.46 g/L, and 2.01 g/L, respectively. PNSB were cultured in modified Ormerod's broth that had initial pH of 6.7. The increasing pH during biohydrogen production of PNSB at 40°C were similar in Fig. 4. The maximum pH of KKU-SN1/1, NM3/1–2, 2M, 14M, and KK(NM)3–21 cultures were 8.47, 8.3, 8.59, 8.34, and 8.26, respectively. The pH increased from the initial value along with the bacterial cell growth and biohydrogen production of PNSB. Between day 7 and day 10 of photo-fermentation, biohydrogen of PNSB declined, while pH rose to more than 8.

The cultivation temperature had an effect on hydrogen production as well as cell growth and substrate degradation efficiency [20, 30]. Several reports have found that temperatures at 40°C have a negative effect on the hydrogen production and bacterial cell growth of *Rhodovulum sulfidophilum* P5 in both wild-type strains and mutants, *Rhodobactor capsulatus* MX01 and the wild type of strain *Rhodobactor capsulatus* SB1003, and *Rhodopseudomonas palustris* CQK 01 [19, 31, 32]. On the other hand, some reports have found thermotolerant PNSB capable of both producing hydrogen and growing at high temperatures. *Rubrivivax gelatinosus* SB24 had potential for hydrogen production at 40°C [33, 34]. *Rhodopseudomonas palustris* ATH 2.1.37 has been acclimated to high temperatures, enabling enhanced hydrogen production under elevated temperature conditions [35]. Previous research by our team found that 14 isolates of thermotolerant photosynthetic biohydrogen producing bacteria could produce hydrogen at 40°C [26].

Growth and biohydrogen production under outdoor conditions

PNSB were cultured in a 300 mL simple bioreactor under outdoor conditions for 20 days to examine photo-fermentation ability for biohydrogen production and bacterial cell growth under natural light. Biohydrogen production of *Rhodopseudomonas* spp. was observed after day 2 until the end of the photo-fermentative biohydrogen production in Fig. 5. The highest cumulative biohydrogen production was 400

mL/L by KKU-SN1/1 followed by 2M, NM3/1–2, 14M, and KK(NM)3–21 were 324 mL/L, 212 mL/L, 187 mL/L, and 173 mL/L, respectively. The highest maximum biohydrogen production rate was obtained from KKU-SN1/1, 3.84 mL/L/h on day 5. The maximum biohydrogen production rate of 2M and NM3/1–2 were 1.94 mL/L/h and 1.20 mL/L/h on day 20. The maximum biohydrogen production rate of 14M and KK(NM)3–21 were 1.02 mL/L/h and 0.83 mL/L/h, respectively, on day 5. Compared to the indoor conditions, NM3/1–2 showed the highest biohydrogen production under indoor conditions as yielded 92 mL/L of cumulative biohydrogen production on day 10 of fermentation, which was 2.4 times lower than the indoor conditions yield. However, by the final day of fermentation, the overall biohydrogen production was nearly identical. Meanwhile, KKUSN1/1 demonstrated a cumulative biohydrogen production that was 1.5 times higher than on day 8 (216 mL/L) and 2.8 times greater than on the final day of fermentation (Fig. 2). This demonstrates that *Rhodopseudomonas* spp., as thermotolerant bacteria, showcased their ability to produce hydrogen in outdoor conditions, even in the face of daytime temperatures reaching as high as 40°C. These findings align with previous experiments.

The bacterial cell growth of PNSB under natural light showed a similar trend (Fig. 6). KKU-SN1/1 and KK(NM)/3–21 growth increased rapidly from the beginning until the end of the experiments. The growth of NM3/1–2, 2M, and 14M quickly increased between day 1 to day 6, after that growing steadily until the end of fermentation. On day 20, KKU-SN1/1 had the highest average dry cell weight of 3.48 g/L, followed by 2M, 14M, KK(NM)3–21 and NM3/1–2, which were 2.43 g/L, 1.98 g/L, 1.81 g/L, and 1.78 g/L, respectively. pH rose during the cultivation of PNSB under outdoor conditions, increasing from 8.0 to 8.8 (Fig. 7). The maximum pH during culture of KKU-SN1/1, NM3/1–2, 2M, 14M, and KK(NM)3–21 were 8.78, 8.51, 8.22, 8.43, and 8.05, respectively; these values were found at the end of the experiments. The higher pH during the culture of *R. pentothenatexigens* KKU-SN1/1 was matched by increased levels of bacterial cell growth and biohydrogen production, which remained detectable at the end of the experiment. At the same time, the fermentation of *R. hawoodiae* entered the pH 8 range near the end of the fermentation, which was slower than in the previous experiment. Thus, biohydrogen production was detected to occur until the end of the experiment.

Temperature variations throughout the day are an obstacle to the hydrogen production of PNSB in outdoor conditions. These fluctuations cause the inhibition of physiological activities, intracellular enzyme activities, and cellular metabolism, resulting in reduced hydrogen production and substrate conversion efficiency [20]. A sustained increase in cell growth may indicate that *Rhodopseudomonas* spp. could be adaptable to unstable temperatures and light conditions. In addition, bacterial cell growth and hydrogen production depend on the intensity of sunlight received in outdoor operation [36]. Previous research reported that light intensity above 5,000 Lux led to lower hydrogen production due to the inhibition of light saturation [37, 38]. This suggests that the biohydrogen production of *Rhodopseudomonas* spp. especially KKUSN1/1 is not negatively impacted by variations in light intensity during the day. Furthermore, *Rhodopseudomonas* spp. demonstrated resilience in the face of fluctuating temperature and light intensity conditions. The biohydrogen production of *Rhodopseudomonas* spp. under natural light conditions proves conducive to commercial applications. This approach minimizes

energy expenditures by eliminating the need for a cooling system and utilizing natural light in the process of biohydrogen production through a light fermentation system.

Scale-up biohydrogen production under outdoor conditions

PNSB were cultured in a 1,500 mL simple bioreactor under outdoor conditions for 20 days. This experiment evaluated the feasibility of scaling up biohydrogen production reactor to improve photo-fermentative biohydrogen production and bacterial cell growth under natural light. The results showed that biohydrogen production of *Rhodopseudomonas* spp. tended to be like that of previous results in Fig. 8. The highest cumulative biohydrogen production was 376 mL/L by KKU-SN1/1 followed by NM3/1–2, KK(NM)3–21, 2M and 14M were 274 mL/L, 273 mL/L, 197 mL/L, and 138 mL/L, respectively. The highest maximum biohydrogen production rate was 1.19 mL/L/h by KKU-SN1/1 on day 3. The maximum biohydrogen production rate of KK(NM)3–21 and NM3/1–2 were 1.06 mL/L/h and 0.98 mL/L/h, respectively, on day 3. The maximum biohydrogen production rate of 14M was 0.70 mL/L/h in day 10. The maximum biohydrogen production rate of 2M was 0.87 mL/L/h on day 18. This indicates that photo-fermentative biohydrogen production by *Rhodopseudomonas* spp. can be successfully expanded for greater capacity.

Bacterial cell growth of *Rhodopseudomonas* spp. which was cultured in a 1,500 mL simple bioreactor under outdoor conditions, behaved similarly to a 300 mL simple bioreactor under outdoor conditions (Fig. 9). Bacterial cell growth of KKU-SN1/1 increased rapidly from day 1 to day 7, steadily from day 10 to the end of fermentation. Bacterial cell growth of NM3/1–2, 2M, 14M, and KK(NM)3–21 increased steadily from day 1 until day 20. At the end of fermentation, KKU-SN1/1 had the highest average dry cell weight of 3.20 g/L, followed by NM3/1–2, KK(NM)3–21, 2M, and 14M, with average dry cell weights of 1.95 g/L, 1.64 g/L, 1.61 g/L, and 1.47 g/L, respectively.

Expansion of hydrogen production via photo-fermentation resulted in decreased output of hydrogen due to the difficulty of maintaining a balance of temperature, light distribution, and mixing between medium and bacterial cells in the bioreactor [5]. In the above-described experiments, expanding the simple bioreactor capacity from 300 mL to 1,500 mL working volume promoted photo-fermentative biohydrogen production under outdoor conditions. In addition, *R. pentothenatexigens* KKU-SN1/1 is an attractive option for photo-fermentative biohydrogen production under outdoor conditions.

Biohydrogen production by CTBS

This study presents the development and implementation of a novel CTBS designed to enhance biohydrogen production efficiency under natural light conditions. The CTBS employs the prolific hydrogen-producing bacteria, *R. pentothenatexigens* KKU-SN1/1, which significantly surpasses laboratory-based biohydrogen production yields and exhibits continuous biohydrogen generation for up to 20 days. Continuous or semi-continuous cultivation techniques are often used to overcome the limitations of batch production time of conventional methods. While these approaches offer potential, challenges arise with large-scale implementation, including increased susceptibility to contamination and

complexities in process control. Additionally, bacteria subjected to numerous fermentation cycles may experience a gradual decline in their efficiency. The CTBS overcomes these limitations by employing a semi-continuous fermentation approach with a crucial modification: the incorporation of multiple bioreactors. This system operates on the principle of continuously adding new bioreactors and removing bioreactors with diminishing yields. This ensures sustained and optimal biohydrogen production rates. By leveraging a model derived from single-batch fermentation data, the CTBS achieves a maximum biohydrogen production rate of 353 mL/L/day on day 20 produced with a 1,500 ml simple bioreactor set of 10 bioreactors (Fig. 10). The production cycle consists of the start phase: 18 days (bioreactor 1 to 10 added sequentially every 2 days), the complete phase: day 20 onwards, and the continuous phase: the first bioreactor replaced with a new one every 2 days.

When the CTBS was operated under outdoor conditions, biohydrogen could be produced continuously for 38 days in the cluster of 10 bioreactors (Fig. 11). The average daily biohydrogen production was 401 mL/L/day from day 12 to 24 and was continuously stable when using the CTBS. The highest daily biohydrogen production was 493 mL/L/day on day 21. Biohydrogen production decreased after 21 days of cultivation because a new bioreactor was not added and the oldest one was removed. Rain, and low light intensity during day 27 led to a significant decrease in biohydrogen production. This indicates that daily weather conditions have a large effect on biohydrogen production under outdoor conditions.

Several studies have shown that the semi-continuous mode is more suitable for hydrogen production by photo-fermentation than other modes due to reduced loss of bacteria and nutrient sources, good system stability, high substrate conversion efficiency, and continuous hydrogen production [39–41]. These findings are supported by our results; the CTBS provides various advantages. It enhances biohydrogen production by eliminating constraints associated with batch production times, resulting in an increased overall biohydrogen yield. Improved efficiency is realized through the continuous rotation of bioreactors, mitigating contamination risks, and facilitating effective troubleshooting. The system is scalable, allowing for the seamless addition of clusters to enhance production capacity as necessary. Its cost-effectiveness is evident, utilizing small bioreactors to optimize light penetration and reduce implementation costs, rendering it suitable for resource-constrained settings. Furthermore, the sustainable design of the CTBS operates effectively under natural light conditions, obviating the requirement for energy-intensive cooling systems.

Conclusions

A PET plastic drinking water bottle can be used as a simple bioreactor for photo-fermentative biohydrogen production. *R. pentothenatexigens* KKU-SN1/1, *R. harwoodiae* NM3/1–2, *R. harwoodiae* 2M, *R. harwoodiae* 14M, and *R. harwoodiae* KK(NM)3–21 were able to grow and produce biohydrogen at 40°C and outdoor conditions with high efficiency. With the ability to tolerate high temperatures and produce biohydrogen under outdoor conditions, *Rhodopseudomonas* spp. has the potential to be used for biohydrogen production under outdoor conditions in tropical countries. This is the first report in which *R.*

harwoodiae and *R. pentothenatexigens* were used to produce biohydrogen at high temperatures and under outdoor conditions in long-term operation.

The CTBS demonstrated the capability to enhance the photo-fermentation of *R. pentothenatexigens* KKU-SN1/1 for biohydrogen production under outdoor conditions, achieving a cumulative biohydrogen production of 9,084 mL over a 38-day period. Notably, the biohydrogen production rate was observed to be 17 times higher compared to traditional batch production methods. The installation of the simple bioreactor within a transparent glass bath filled with water effectively regulates temperature fluctuations, ensuring ideal conditions for biohydrogen generation. The CTBS represents a significant advancement in biohydrogen production technology, especially for developing countries. Its simplicity, affordability, and sustainability make it a viable solution for promoting clean energy solutions in resource-limited environments. Additionally, the ability of the system to leverage natural light and minimize energy consumption aligns with global sustainability goals. This research paves the way for further investigations into optimizing CTBS design and performance, ultimately contributing to the development of a more sustainable and equitable energy future.

Declarations

Conflict of Interest

The authors declare no competing interests.

Author Contribution

Netchanok Punriboon: conceptualization, methodology, formal analysis and investigation, writing-original draft preparation. Jutaporn Sawaengkaew: writing-review and editing. Polson Mahakhan: supervision and writing-review and editing. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Research Fund for Supporting Lecturer to Admit High Potential Student to Study and Research on His Expert Program Year 2020, Khon Kaen University, Thailand (Grant No. 631T221-C); and the Fundamental Fund of Khon Kaen University and the National Science, Research, and Innovation Fund (NSRF), Thailand. The authors are grateful for the advice from Prof. Alissara Reungsang and the support from the Student Development Camp "GS KKU Manuscript Camp", Khon Kaen University, Thailand.

References

1. Mahapatra S, Kumar D, Singh B, Sachan PK (2021) Biofuels and their sources of production: A review on cleaner sustainable alternative against conventional fuel, in the framework of the food and energy nexus. Energy Nexus 4: 100036
2. Li B, Haneklaus N (2021) The role of renewable energy, fossil fuel consumption, urbanization and economic growth on CO_2 emissions in China. Energy Reports 7: 783–791
3. Verma D, Kumar NR, Subudhi S (2020) Isolation and characterization of a novel photoheterotrophic strain '*Rubrivivax benzoatilyticus* TERI-CHL1': Photo fermentative hydrogen production from spent effluent. Int J Hydrogen Energy 45: 14245–14254
4. Savasturk D, Kayahan E, Koku H (2018) Photofermentative hydrogen production from molasses: Scale-up and outdoor operation at low carbon-to-nitrogen ratio. Int J Hydrogen Energy 43: 11676–11687
5. Tarhan C, Cil, MA (2021) A study on hydrogen, the clean energy of the future: Hydrogen storage methods. J Energy Storage 40: 102676
6. Morsy FM, Elbahloul Y, Elbadry M (2019) Photoheterotrophic growth of purple non-sulfur bacteria on Tris Acetate Phosphate Yeast extract (TAPY) medium and its hydrogen productivity in light under nitrogen deprivation. Int J Hydrogen Energy 44: 9282–9290
7. Elkahlout K, Sagir E, Alipour S, Koku H, Gunduz U, Eroglu I, Yucel M (2018) Long-term stable hydrogen production from acetate using immobilized *Rhodobacter capsulatus* in a panel photobioreactor. Int J Hydrogen Energy 44: 18801–18810
8. Hakobyan L, Gabrielyan L, Trchounian A (2019) Biohydrogen by *Rhodobacter sphaeroides* during photo-fermentation: Mixed vs. sole carbon sources enhance bacterial growth and H_2 production. Int J Hydrogen Energy 44: 674–679
9. Miandad R, Rehan M, Ouda OKM, Khan, MZ, Shazad K, Ismail IMI, Nizami AS (2017) Waste-to-Hydrogen Energy in Saudi Arabia: Challenges and Perspectives. In: Singh A, Rathore D (eds) Biohydrogen Production: Sustainability of Current Technology and Future Perspective. New Delhi, pp 237–252
10. Isaac M, Eliane G, German B (2016) A mechanistic model supported by data-based classification models for batch hydrogen production with an immobilized photo-bacteria consortium. Int J Hydrogen Energy 41: 22802–22811
11. Imhoff JF (2006) The Phototrophic Alpha-Proteobacteria. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (eds) The Prokaryote. New York, pp 41–64
12. Kapdan IK, Kargi F (2006) Bio-hydrogen production from waste materials. Enzyme Microb Technol 38: 569–582
13. Das D, Veziroglu TN (2011) Advances in biological hydrogen production processes. Int J Hydrogen Energy 33: 6046–6057
14. Stevens P, Vertonghen C, De VP, De LJ (1984) The effect of temperature and light intensity on hydrogen gas production by different *Rhodopseudomonas capsulata* strains. Biotechnol Lett, 6 (5): 277–282

15. Basak N, Das D (2007) The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. World J Microb Biotechnol 23: 31–42
16. Sevinc P, Gunduz U, Eroglu I, Yucel M (2012). Kinetic analysis of photosynthetic growth, hydrogen production and dual substrate utilization by *Rhodobacter capsulatus*. Int J Hydrogen Energy 37 (21): 16430–16436
17. Basak N, Jana AK, Das D, Saikia D (2014) Photofermentative molecular biohydrogen production by purple-non-sulfur (PNS) bacteria in various modes: The present progress and future perspective. Int J Hydrogen Energy 39: 6853–6871
18. Laocharoen S, Reungsang A (2014) Isolation, characterization and optimization of photo-hydrogen production conditions by newly isolated *Rhodobacter sphaeroides* KKU-PS5. Int J Hydrogen Energy 39: 10870–10882
19. Wei X, Feng J, Cao W, Li Q, Guo L (2021) Photo-biological hydrogen production by a temperature-tolerant mutant of *Rhodobacter capsulatus* isolated by transposon mutagenesis. Bioresour Technol 320: 124286
20. Reungsang A, Zhong N, Yang Y, Sittijunda S, Xia A, Liao Q (2018) Hydrogen from Photo Fermentation. In: Liao Q, Chang J, Herrmann C, Xia A (eds) Bioreactors for Microbial Biomass and Energy Conversion. Singapore, pp 221–317
21. Wakayama T, Miyake J (2002) Light shade bands for the improvement of solar hydrogen production efficiency by *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 27: 1495–1500
22. Androga DD, Ozgur E, Eroglu I, Gunduz U, Yucel M, Eroglu I (2011) Factors affecting the longterm stability of biomass and hydrogen productivity in outdoor photofermentation. Int J Hydrogen Energy 36: 11369–11378
23. Sagir E, Alipour S, Elkahlout K, Koku H, Gunduz U, Eroglu I, Yucel M (2018) Biological hydrogen production from sugar beet molasses by agar immobilized *R. capsulatus* in a panel photobioreactor. Int J Hydrogen Energy 43: 14987–14995
24. Kumar B., Ramprasad EVV, Sasikala C, Ramana CV (2013) *Rhodopseudomonas pentothenatexigens* sp. nov. and *Rhodopseudomonas thermotolerans* sp. nov., isolated from paddy soils. Int J Syst Evol Microbiol 63: 200–207
25. Ramana VV, Chakravarthy SK, Raj PS, Kumar BV, Shobha E, Ramaprasad EVV, Sasikala C, Ramana CV (2012) Descriptions of *Rhodopseudomonas parapalustris* sp. nov., *Rhodopseudomonas harwoodiae* sp. nov. and *Rhodopseudomonas pseudopalustris* sp. nov., and emended description of *Rhodopseudomonas palustris*. Int J Syst Evol Microbiol 62: 1790–1798
26. Phankhamla P, Sawaengkaew J, Buasri P, Mahakhan P (2014) Biohydrogen production by a novel thermotolerant photosynthetic bacterium *Rhodopseudomonas pentothenatexigens* strain KKU-SN1/1. Int J Hydrogen Energy 39: 15424–15432
27. Lakum A, Sawaengkaew J, Mahakhan P (2012) Isolation and characterization of bio-hydrogen by photosynthetic bacteria isolated from the northeastern of Thailand. International Conference on Microbial Taxonomy, Basic and Applied Microbiology, Khon Kaen, Thailand

28. Ormerod JC, Ormerod KS, Gest H (1961) Light dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria: relationship with nitrogen metabolism. Arch Biochem Biophys 94: 449–63
29. Mabutyana L, Pott RW (2021) Photo-fermentative hydrogen production by *Rhodopseudomonas palustris* CGA009 in the presence of inhibitory compounds. Int J Hydrogen Energy 46: 29088–29022
30. He D, Bultel Y, Magnin JP, Willison JC (2006) Kinetic analysis of photosynthetic growth and photohydrogen production of two strains of *Rhodobacter capsulatus*. Enzyme Microboal Technol 38: 253–259
31. Cai J, Wang G (2014) Photo-biological hydrogen production by an acid tolerant mutant of *Rhodovulum sulfiphilum* P5 generated by transposon mutagenesis. Bioresour Technol 154: 254–259
32. Wang YZ, Liao Q, Zhu X, Tian X, Zhang C (2010) Characteristics of hydrogen production and substrate consumption of *Rhodovulum palustris* CQK in an immobilized cell photobioreactor. Bioresour Technol 101: 4034–4041
33. Mahakhan P, Chobvijuk C, Ngmjarearnwong M, Trakulnalermsai S, Bucke C, Svasti J, Kanlayakritd W, Chitradon L (2005) Molecular hydrogen production by a thermotolerant *Rubrivivax gelatinosus* using raw cassava starch as an electron donor. Science Asia 31: 415-424.
34. Channarong W, Ngamjarearnwong M, Teerapatsakul C, Chitradon L (2018) A Complete System for Renewable Utilization of Cassava to Biohydrogen Coupled with Biomethane as Zero-Waste Management. Chiang Mai Journal of Science 45: 1214–1224
35. Toit JP, Pott RW (2021) Heat-acclimatised strains of *Rhodopseudomonas palustris* reveal higher temperature optima with concomitantly enhanced biohydrogen production rates. Int J Hydrogen Energy 46: 11564–11572
36. Androga DD, Ozgur E, Eroglu I, Gunduz U, Yucel M (2012) Photofermentative Hydrogen Production in Outdoor Conditions. In: Minic D (ed) Hydrogen Energy-Challenges and Perspectives. pp 77–120
37. Mishra P, Thakur S, Singh L, Wahid ZA, Sakinah M (2016) Enhanced hydrogen production from palm oil mill effluent using two stage sequential dark and photo fermentation. Int J Hydrogen Energy 41: 18431–18440
38. Lu C, Zhang Z, Ge X, Wang Y, Zhou X, You X, Liu H, Zhang Q (2016) Bio-hydrogen production from apple waste by photosynthetic bacteria HAU-M1. Int J Hydrogen Energy 41: 13399–13407
39. Zhang C, Su H, Tan T (2010) Batch and semi-continuous anaerobic digestion of food waste in a dual solid-liquid system. Bioresour Technol 145: 10–16
40. Wu YN, Wen HQ, Zhu JN, Ding J, Ren NQ, Liu BF (2016) Best mode for photo-fermentation hydrogen production: The semi-continuous operation. Int J Hydrogen Energy 41: 16048–16054
41. Li Y, Zhang Z, Xia C, Jing Y, Zhang Q, Lia S, Zhu S, Jin P (2020) Photo-fermentation biohydrogen production and electrons distribution from dark fermentation effluents under batch, semi-continuous and continuous modes. Bioresour Technol 311: 123549

Figures

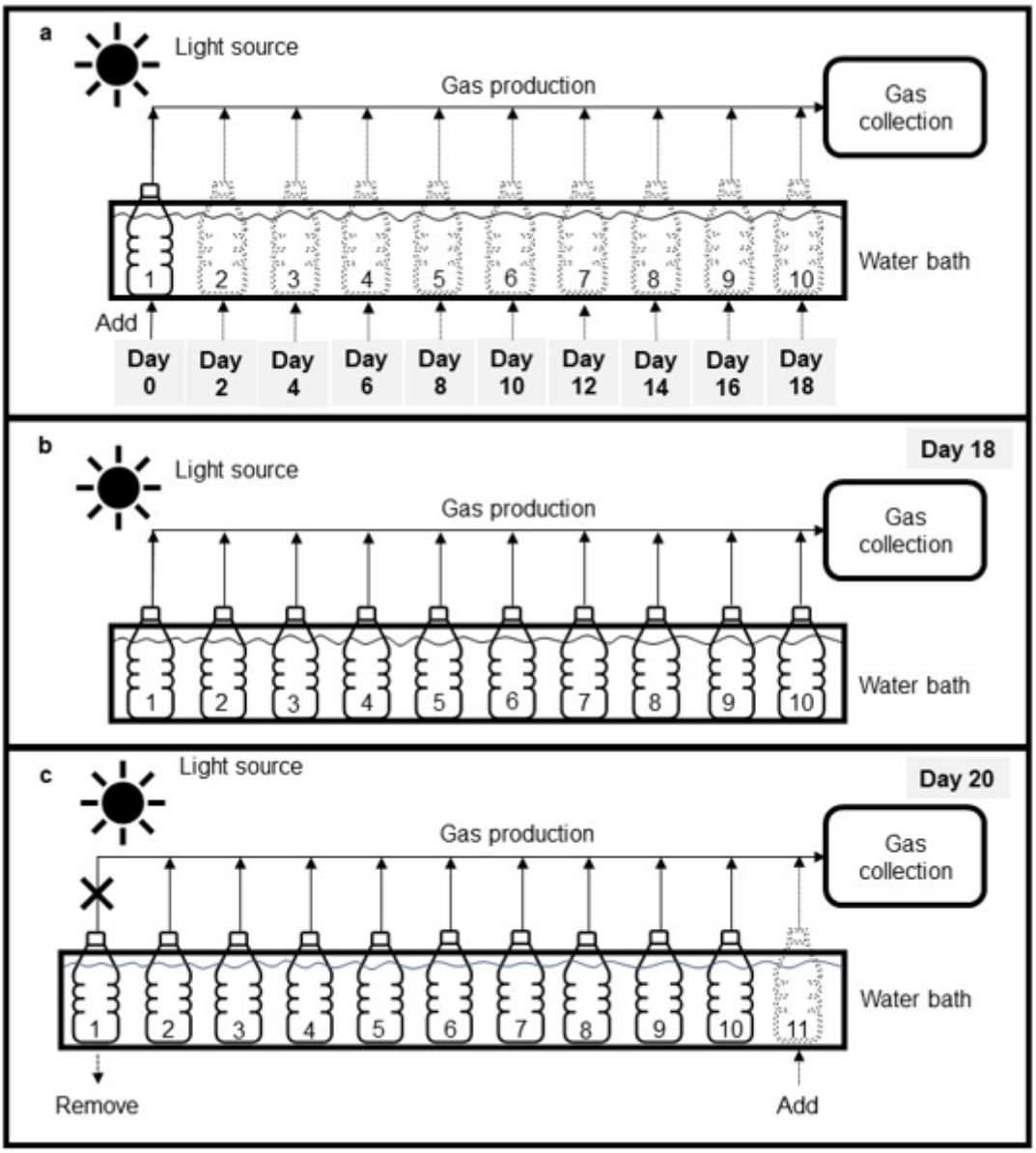

Figure 1

Biohydrogen production by cluster of ten bioreactors system (CTBS) consists of 3 phases of operation: the start phase (a), the complete phase (b), and the continuous phase (c)

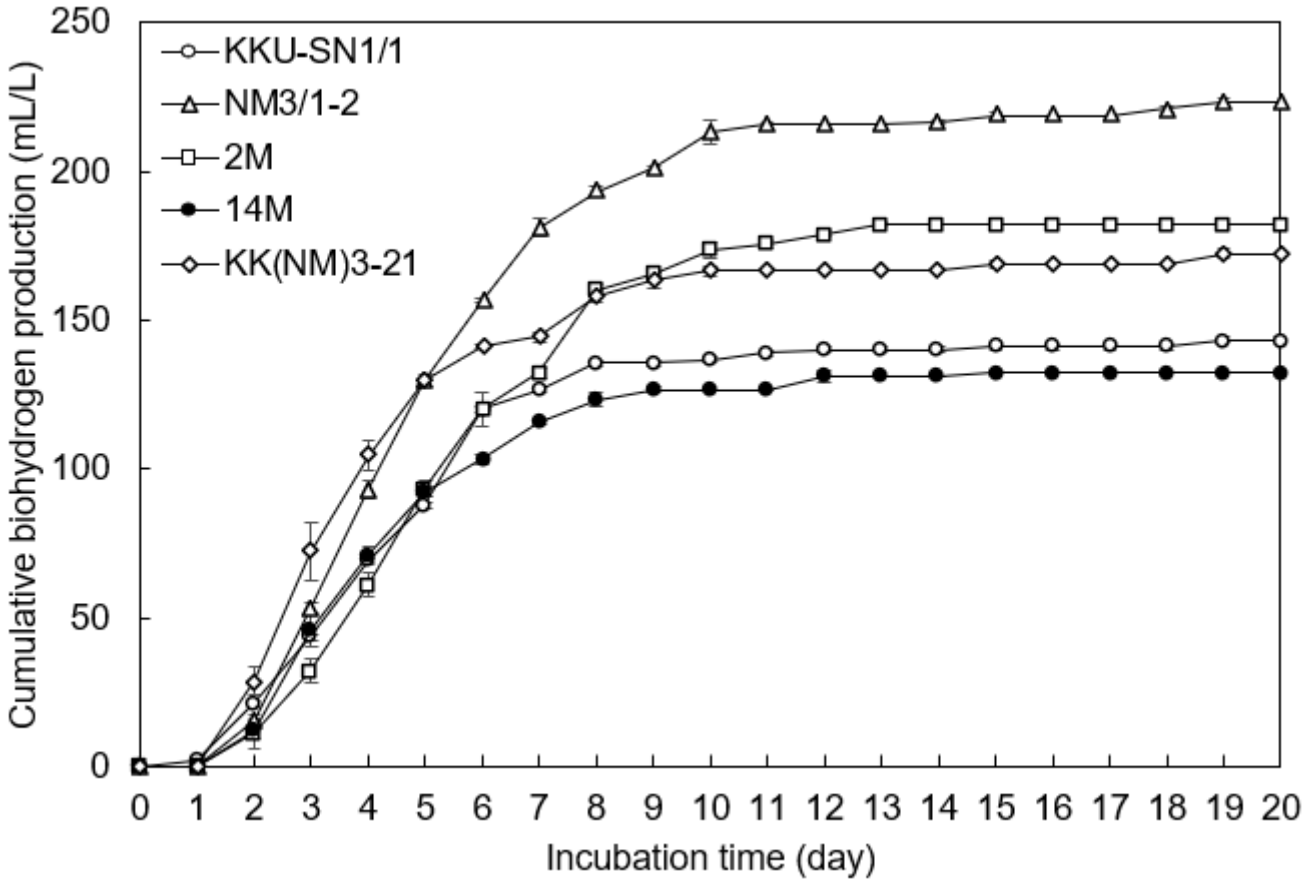

Figure 2

Cumulative biohydrogen production by *Rhodopseudomonas* spp. in a 300 mL modified Ormerod's medium incubated under an illumination of 10,000 Lux at 40 °C. Error bars represent the standard error of the means for replicates

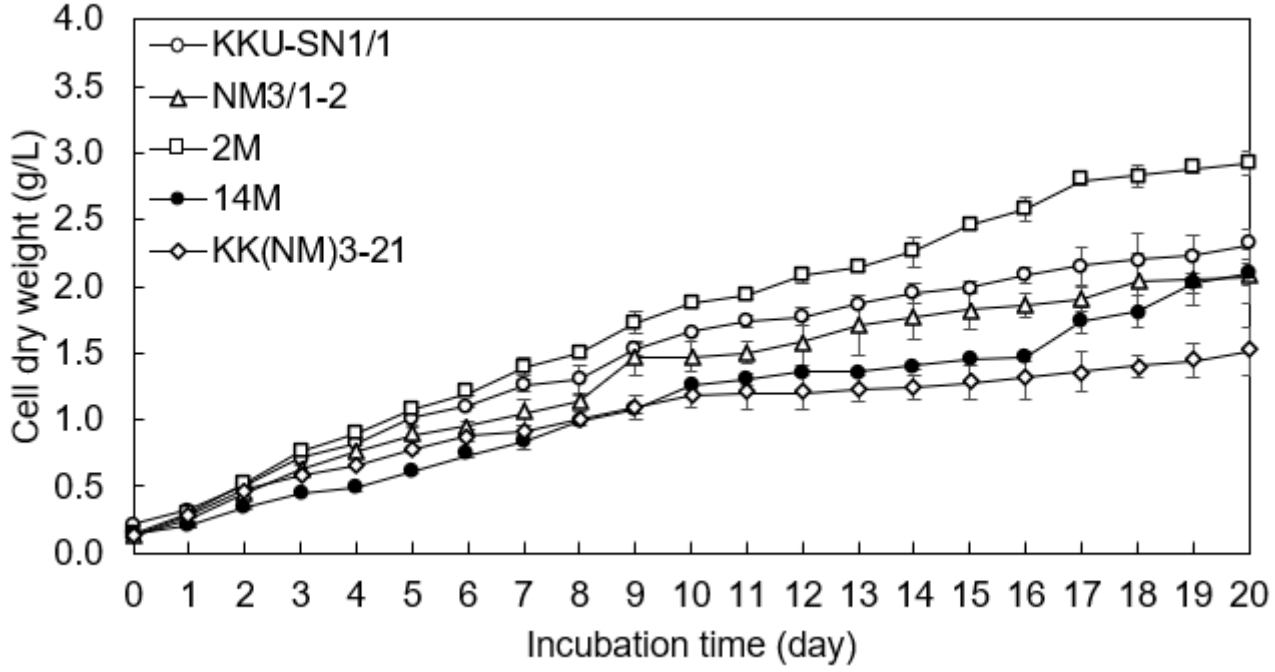

Figure 3

Bacterial cell growth during photo-fermentation of *Rhodopseudomonas* spp. in a 300 mL modified Ormerod's medium incubated under an illumination of 10,000 Lux at 40 °C. Error bars represent the standard error of the means for replicates

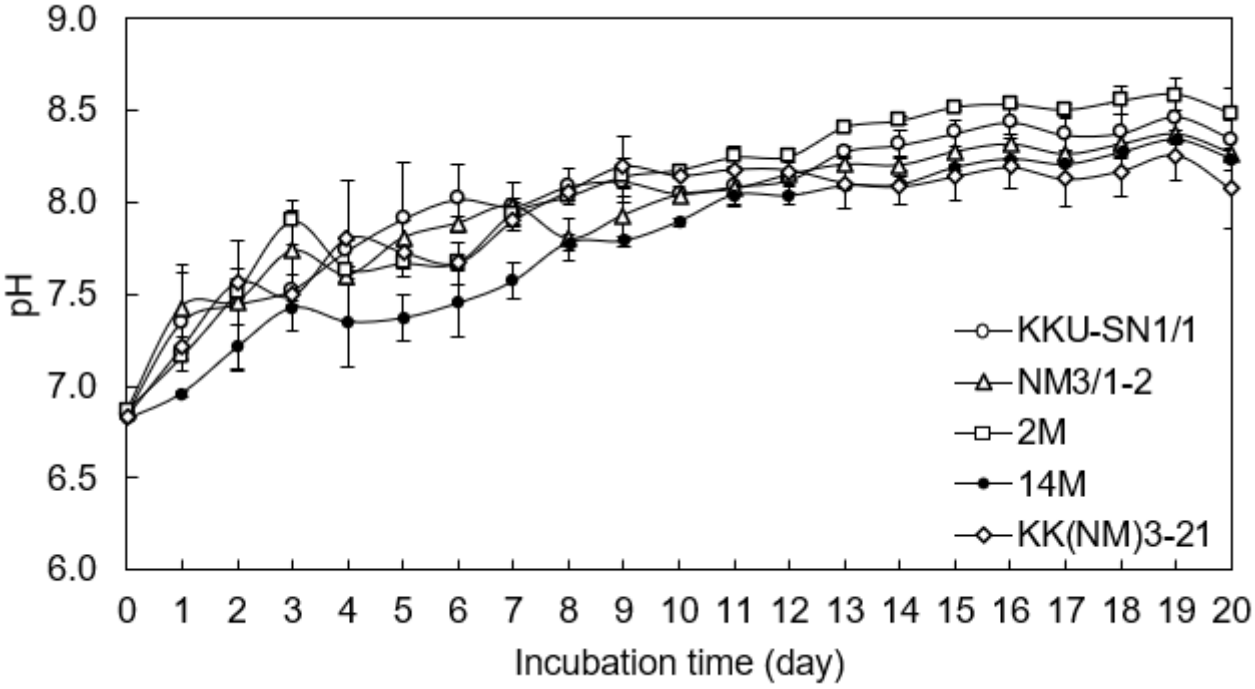

Figure 4

The pH during photo-fermentation of *Rhodopseudomonas* spp. in a 300 mL modified Ormerod's medium incubated under an illumination of 10,000 Lux at 40 °C. Error bars represent the standard error of the means for replicates

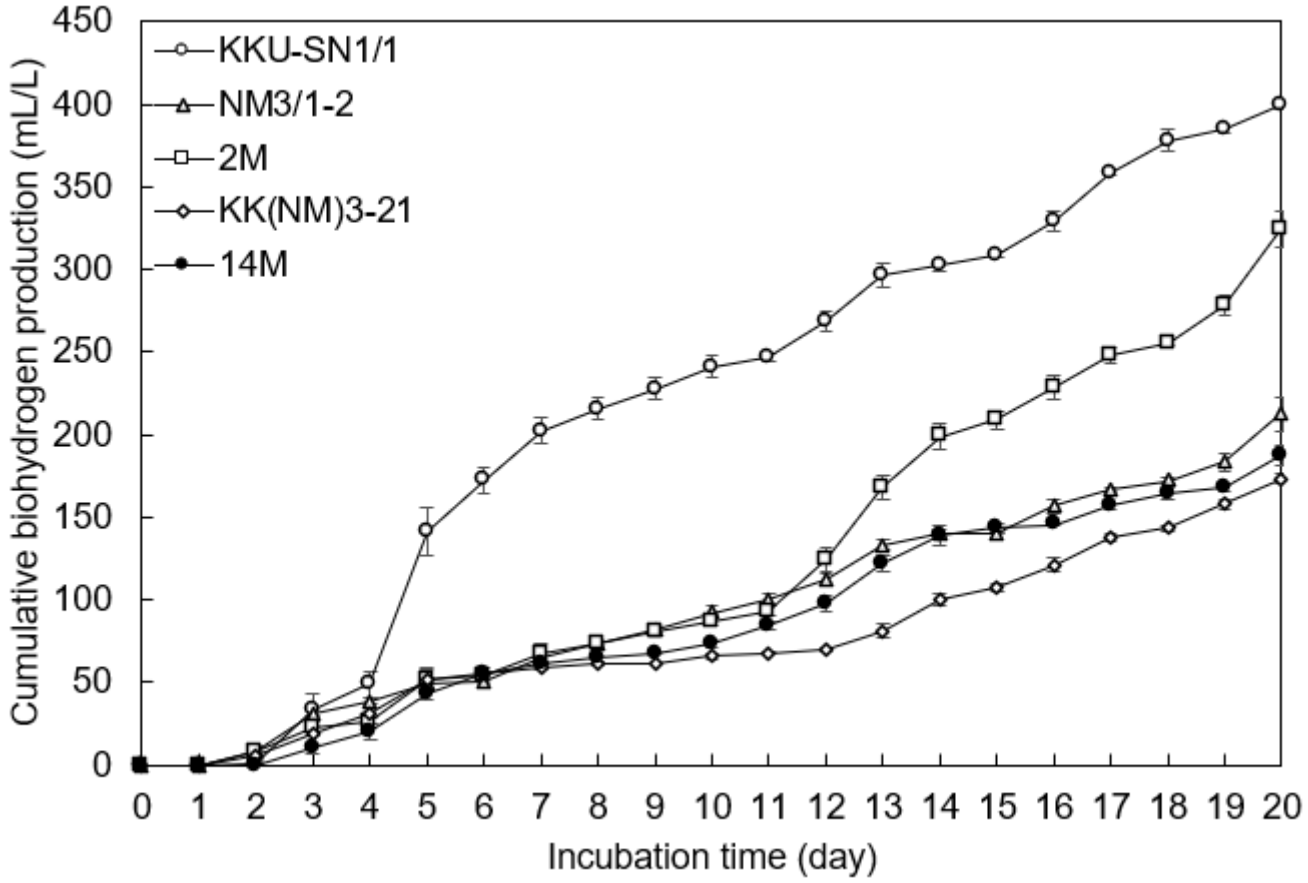

Figure 5

Cumulative biohydrogen production by *Rhodopseudomonas* spp. in a 300 mL modified Ormerod's medium incubated under outdoor conditions. Error bars represent the standard error of the means for replicates

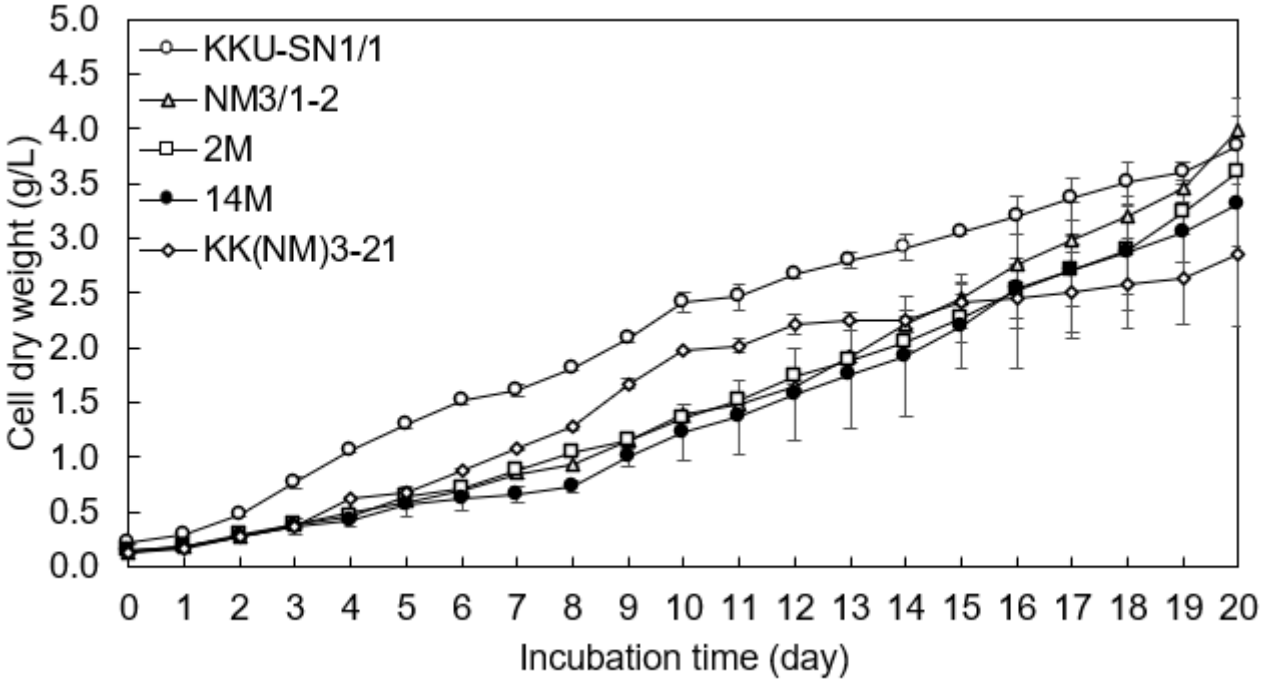

Figure 6

Bacterial cell growth of *Rhodopseudomonas* spp. in 300 mL modified Ormerod's medium incubated under outdoor conditions. Error bars represent the standard error of the means for replicates

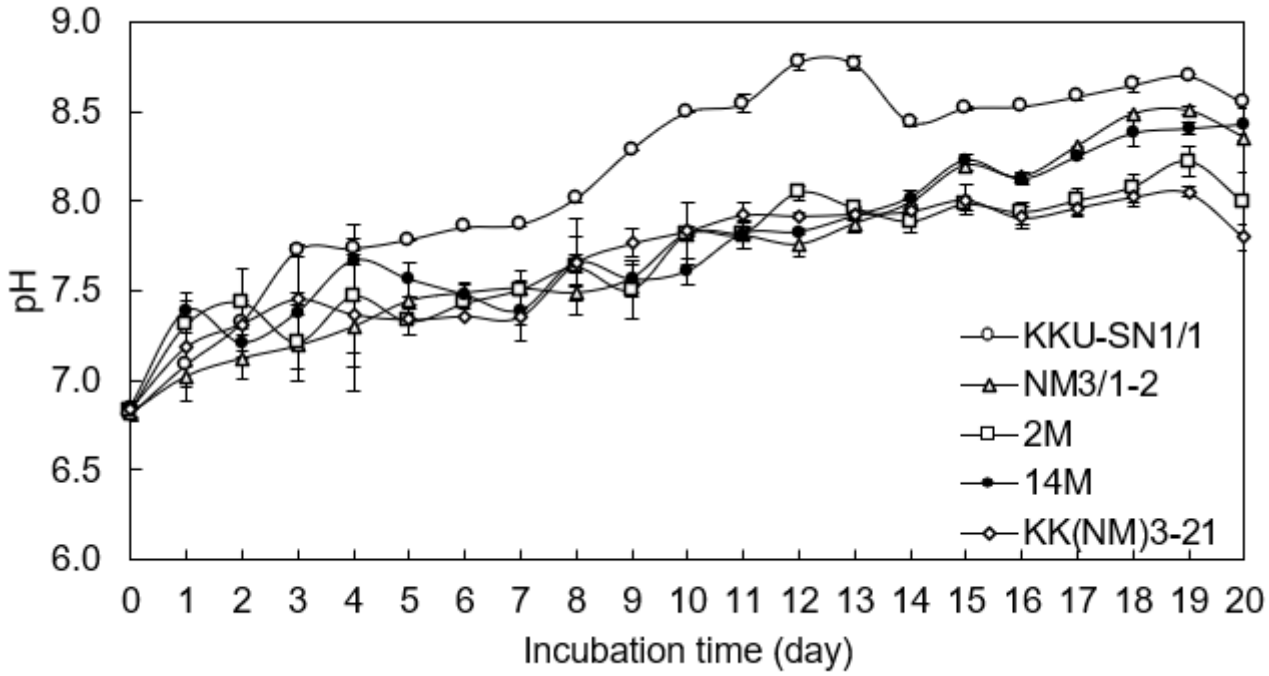

Figure 7

The pH during fermentation of *Rhodopseudomonas* spp. in 300 mL modified Ormerod's medium incubated under outdoor conditions. Error bars represent the standard error of the means for replicates

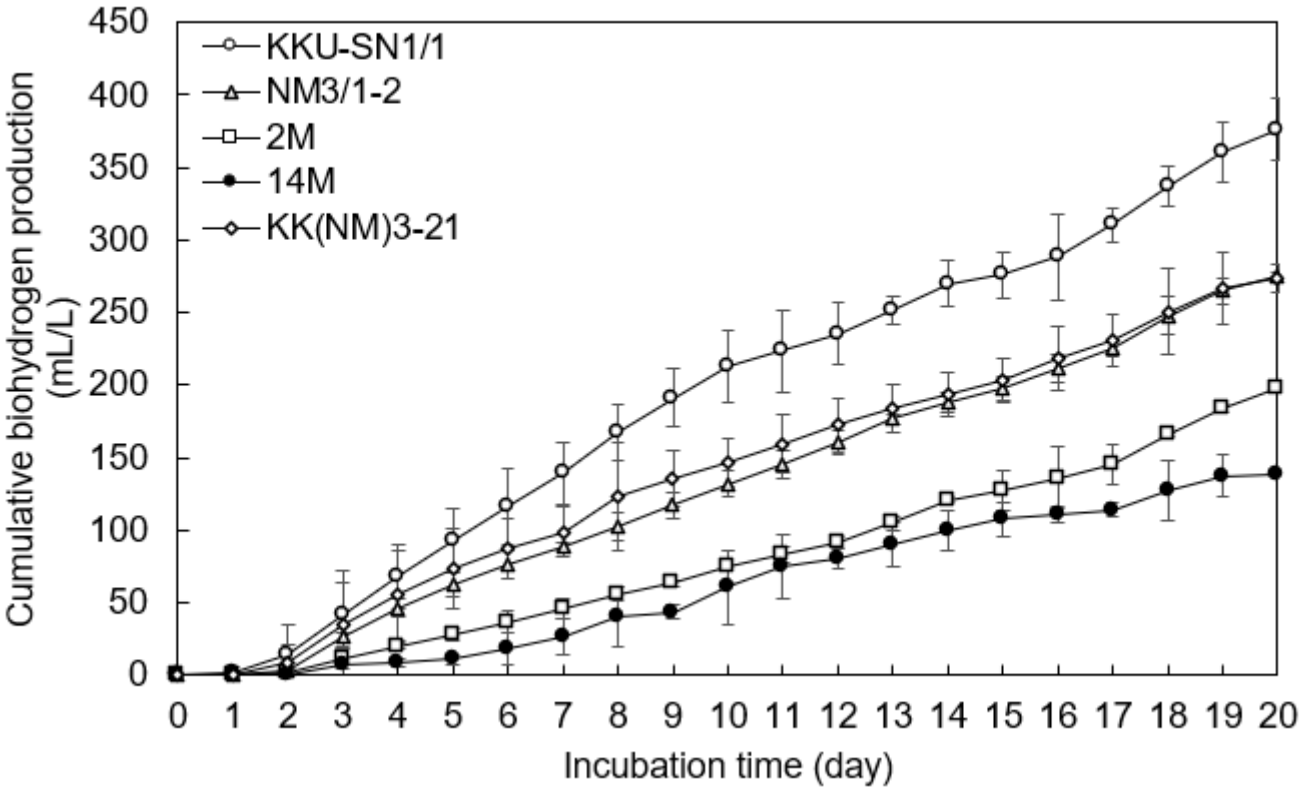

Figure 8

Cumulative biohydrogen production by *Rhodopseudomonas* spp. in a 1,500 mL modified Ormerod's medium incubated under outdoor conditions. Error bars represent the standard error of the means for replicates

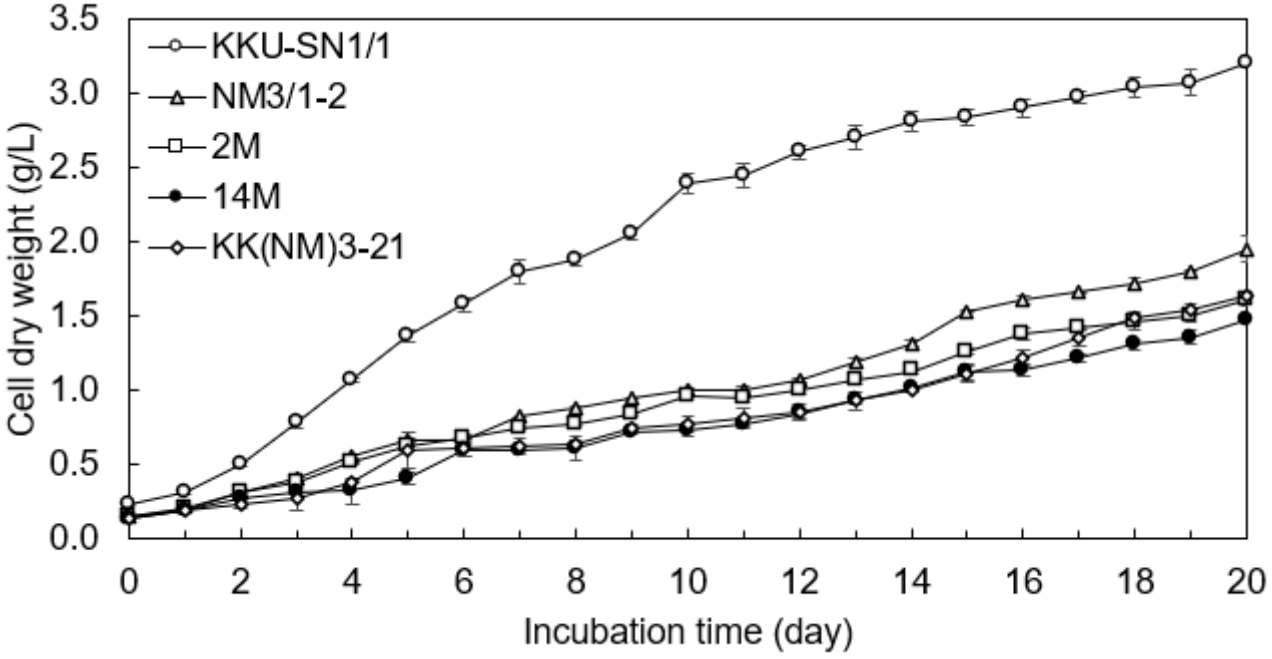

Figure 9

Bacterial cell growth of *Rhodopseudomonas* spp. in 1,500 mL modified Ormerod's medium incubated under outdoor conditions. Error bars represent the standard error of the means for replicates

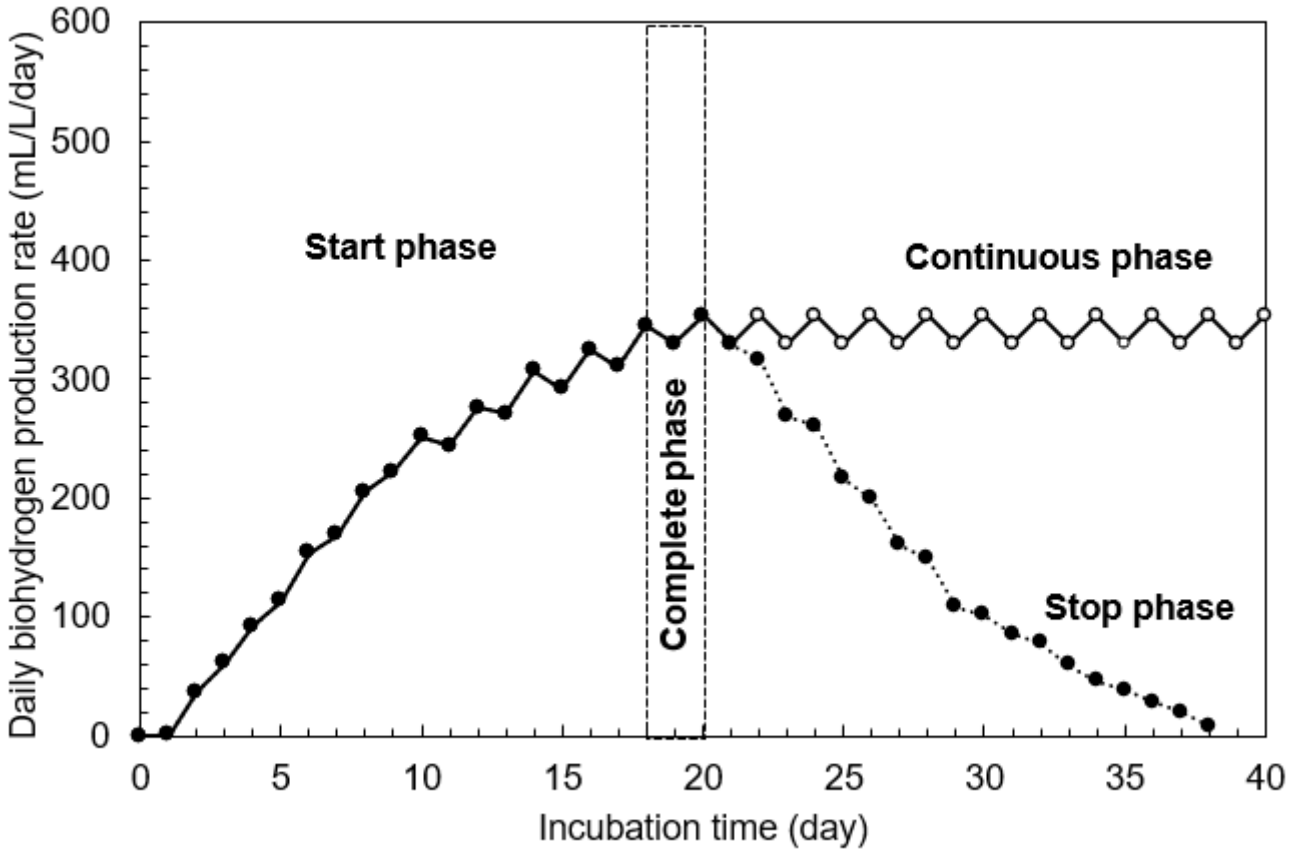

Figure 10

Simulating daily biohydrogen production involves continuous simulations using CTBS from the start phase to continuous phase (●) and the simulation of daily biohydrogen production using a cluster of 10 bioreactor systems over a 38-day period from the start phase to stop phase (●)

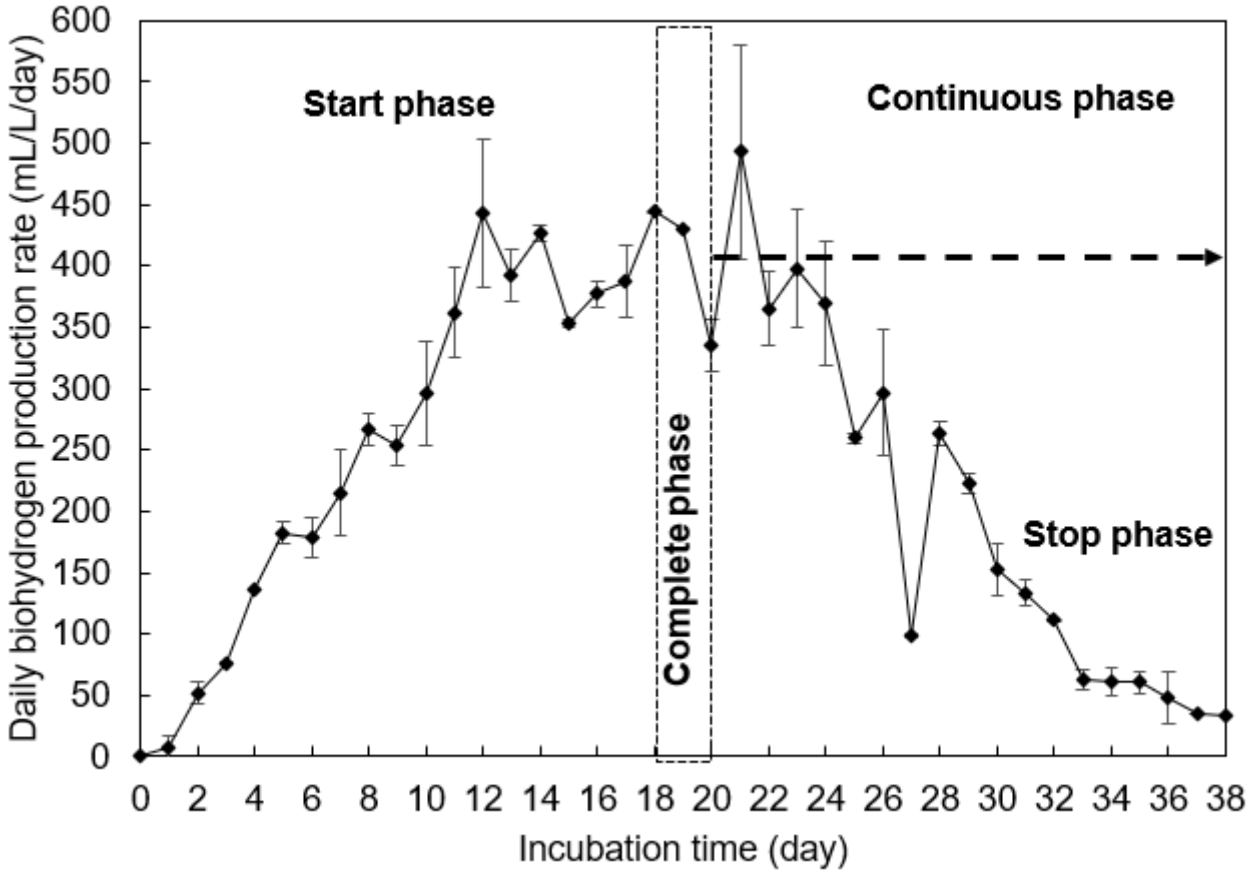

Figure 11

Daily biohydrogen production by *R. pentothenatexigens*KKU-SN1/1 by CTBS incubated under outdoor conditions for 38 days (♦). Error bars represent the standard error of the means for replicates