Photo-fermentational hydrogen production of *Rhodobacter* sp. KKU-PS1 isolated from an UASB reactor

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**Background:** In this study, the detection of nifH and nifD by a polymerase chain reaction assay was used to screen the potential photosynthetic bacteria capable of producing hydrogen from five different environmental sources. Efficiency of photo-hydrogen production is highly dependent on the culture conditions. Initial pH, temperature and illumination intensity were optimized for maximal hydrogen production using response surface methodology with central composite design.

**Results:** *Rhodobacter* sp. KKU-PS1 (GenBank Accession No. KC478552) was isolated from the methane fermentation broth of an UASB reactor. Malic acid was the favored carbon source while Na-glutamate was the best nitrogen source. The optimum conditions for simultaneously maximizing the cumulative hydrogen production (H\(_{\max}\)) and hydrogen production rate (R\(_m\)) from malic acid were an initial of pH 7.0, a temperature of 25.6°C, and an illumination intensity of 2500 lx. H\(_{\max}\) and R\(_m\) levels of 1264 ml H\(_2\)/l and 6.8 ml H\(_2\)/L-h were obtained, respectively. The optimum initial pH and temperature were further used to optimize the illumination intensity for hydrogen production. An illumination intensity of 7500 lx gave the highest values of H\(_{\max}\) (1339 ml H\(_2\)/l) and R\(_m\) (12.0 ml H\(_2\)/L-h) with a hydrogen yield and substrate conversion efficiency of 3.88 mol H\(_2\)/molmalate and 64.7%, respectively.

**Conclusions:** KKU-PS1 can produce hydrogen from at least 8 types of organic acids. By optimizing pH and temperature, a maximal hydrogen production by this strain was obtained. Additionally, by optimizing the light intensity, R\(_m\) was increased by approximately two fold and the lag phase of hydrogen production was shortened.

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1. Introduction

Alternative energy is one of the solutions to environmental problems such as global warming and climate change. Among the alternative energies available, hydrogen has received increasing attention due to its environmentally friendly characteristics and high energy content [1]. Biologically, hydrogen can be produced by dark fermentation, photo-fermentation, biophotolysis, and microbial electrolysis [2]. Dark fermentation utilizes various substrates to produce hydrogen at a high rate in which volatile fatty acids (VFAs) are produced as the main soluble metabolites. VFAs can accumulate in the effluent that requires additional treatment before being released into the environment. In addition, the imbalance between acidogens and methanogens results in digestion failure due to the accumulation of VFAs with an associated drop in pH [3]. However, VFAs can be utilized by photosynthetic bacteria to produce hydrogen with a high hydrogen yield (HY) (i.e., approximately 50% of theoretical value (4–6 mol H\(_2\)/mol\(\text{glucose}\)) [4,5]. For this reason, a search for microorganisms that efficiently produce hydrogen from VFA containing wastes is needed. Apart from reducing COD loading, hydrogen can also be produced from these VFA containing wastes. Examples of wastes containing VFAs are those from the wineries, distilleries and beverage industries [6,7] as well as the effluent from hydrogen production process by dark fermentation.

Among the bio-hydrogen producers, the purple non-sulfur photosynthetic bacteria (PNSB) are regarded as effective in producing hydrogen from different kinds of substrates containing VFAs and sugar [8]. *Rhodobacter sphaeroides* KKU-PS5 used malate [9] and *R. sphaeroides* KD131 used succinate to produce hydrogen [10]. Lactate was reported as the substrate for hydrogen production by *R. sphaeroides* KD131 [11]. Acetate and butyrate were used by *R. sphaeroides* O.U.001 [12] and *Rhodopseudomonas palustris* WP3-5 [13] to produce hydrogen, respectively. Mixed VFAs consisting of acetate, propionate and butyrate were used by *Rhodopseudomonas capsulata* to produce hydrogen [14]. Additionally, the effluent from dark fermentation of hydrogen was used as a substrate to produce hydrogen by *R. sphaeroides* NRRL-B1772 [15] and *R. sphaeroides* RS [16]. Not only VFAs but also other carbon sources such as glucose were used by *palustris R*. CQK 01 to produce hydrogen...
2. Materials and methods

2.1. Source of microorganisms

Hydrogen-producing PNSB were isolated from aquatic and wastewater treatment facilities. Samples were taken from the following three aquatic environments: East Sri-Than Lake (EST), Khon Kaen University, Khon Kaen, Thailand; West Sri-Than Lake (WST), Khon Kaen University, Khon Kaen, Thailand; and Songkhla Lake (SK), following three aquatic environments: East Sri-Than Lake (EST), Khon Kaen University, Khon Kaen, Thailand; West Sri-Than Lake (WST), Khon Kaen University, Khon Kaen, Thailand; and Songkhla Lake (SK), Kaen University, Khon Kaen, Thailand; and Songkhla Lake (SK), Kaen University, Khon Kaen, Thailand.

In this research, we attempted to isolate PNSB capable of producing hydrogen from different environmental sources using the PCR assay to detect nifH and nifD genes. Subsequently, the newly isolated strains were identified, phylogenetically classified, and screened for carbon and nitrogen utilization. The key culture conditions affecting bio-hydrogen production included initial pH and incubation temperature. These variables were optimized by RSM with CCD in order to achieve maximal hydrogen production and HPR. Furthermore, the effects of illumination intensity on hydrogen production were determined at the optimal initial pH and incubation temperature. The isolate obtained from this study can potentially be used to treat VFA containing wastewaters using an integrated system of dark-fermentative bacteria and PNSB.

2.2. Isolation of the microorganisms

PNSB were enriched and isolated by successive anaerobic cultivation under continuous illumination. Briefly, approximately 2 mL of each sample was inoculated into 18 mL of a basal medium which consisted of 2 g/L of α-malic acid as a carbon source, 0.36 g/L of sodium glutamate as a nitrogen source, and 1 mL/L of trace elements [36]. The basal medium consisted of 0.5 g/L of KH₂PO₄, 0.4 g/L of MgSO₄ × 7H₂O, 0.4 g/L of NaCl, 0.05 g/L of CaCl₂ × 2H₂O, and 1.0 g/L of yeast extract. The trace elements consisted of 68.2 mg/L of ZnCl₂, 72.7 mg/L of MnSO₄ × H₂O, 62 mg/L of H₃BO₃, 190 mg/L of CoCl₂ × 6H₂O, 17.04 mg/L of CuCl₂ × 2H₂O, 23.77 mg/L of NiCl₂ × 6H₂O, 62.76 mg/L of Na₂MoO₄ × 2H₂O, and 5.24 mg/L of EDTA-Na₂ × 2H₂O. The pH of the medium was adjusted to 6.8 with the addition of NaOH pellets before autoclaving. Enrichment of the culture was conducted anaerobically at room temperature (32 ± 2°C) under an illumination intensity of 2500 lx using light-emitting diode (LED) lamps (E27 Corn-1205, epistar). Samples were horizontally shaken at 150 rpm. After 48 h of incubation, 2 mL of culture was transferred into another 18 mL of the basal medium. After three cycles of subculture, a single loop of the liquid culture was streaked onto basal medium agar plates (1.5% w/v agar) and incubated in an anaerobic jar under lighted conditions. After 7 d of incubation, red or pink colonies were selected and re-streaked three times. In this way, pure cultures were obtained. Each putative PNSB strain was transferred into serum bottles containing basal medium and incubated as described above.

2.3. DNA extraction and amplification of nifH and nifD genes by PCR

Total genomic DNA was extracted from 2 mL cultures generated anaerobically under continuous illumination at 2500 lx for 2 d. The total genomic DNA of selected isolates was extracted using the method described by Tan et al. [25]. PCR amplification of 16S rDNA was performed using the same conditions for all of the strains. The specific primers for the detection of the nifH and nifD genes in the genome of *palustris Rp* (GenBank NC005296) were designed by Tan et al. [25]. The nifH forward primer NH1 (5′- ACT CCA CCC TGT TGA TCC TC-3′) and nifH reverse primer NH2 (5′- CGC AGC ACG TCA TAG GAC-3′) target a fragment of 253 bp which corresponded to the genomic location, 5,203,393 - 5,203,934. The nifD forward primer ND1 (5′- TGC TAC CCG TGC ATG AAC TA-3′) and nifD reverse primer ND2 (5′- AAC CCG TCG TAG CCA TGA TA-3′) target fragment of 545 bp which corresponded to location 5,203,390 - 5,203,934. PCR amplification was performed in 25 μL aliquots containing 0.5 μL of the DNA template, 1.0 μL of primers (25 μM), 12.5 μL of 2XPCR Master Mix #K0171 (Fermentas), and 11.0 μL of distilled water. The PCR was carried out on a 2 × 2 thermal cycler (Thermo IEC Inc., Milford, MA, USA) following the method of Tan et al. [25]. Separation of the PCR products was achieved by agarose gel electrophoresis for 25 min at 90 V on a 0.8% (w/v) agarose gel in 1X TBE (Tris-borate EDTA) buffer. The gel was stained with ethidium bromide for 15 min and visualized on a UV transilluminator (Dolphin-Doc, Wealtec, Taiwan).

2.4. Strain identification and phylogenetic analysis

The 16S rRNA gene sequences were amplified by PCR using a universal primer set composed of the forward primer PA (5′-AGA GTT TGA TCC TGG CTC AG-3′), corresponding to positions 19–38. The reverse primer PH (5′-AAG GAG GTG ATC CAG CCG CA-3′), corresponding to positions 1541–1561 was also used [37]. The PCR protocol followed the method of Khamtib et al. [38]. The PCR products were detected using the PCR assay described above.
were purified using a gel extraction method and sequenced using the BigDye® Terminator cycle sequencing kit, Version 3.1 (1st BASE Laboratories SdnBhd, Seri Kembangan, Malaysia) in accordance with the manufacturer’s instructions. The partial 16S rRNA gene sequences were matched using DNA Baser Version 4.12 and identified by searching in GenBank using BLAST tools [39]. Subsequently, the sequences of reference microorganisms obtained from GenBank were aligned using Clustal X [40]. A phylogenetic tree was created to assess evolutionary distance using the neighbor-joining method [41]. Bootstrapping analysis [42] of 1000 re-samplings was performed to measure the confidence of the tree topologies using Mega 4 Version 4.0.2.

2.5. Bio-hydrogen production of the isolated PNSB strains using different carbon and nitrogen sources

The capacity of the KKU-PS1 strain to utilize different carbon and nitrogen sources for bio-hydrogen production was investigated in batch fermentation. The different carbon sources tested included malate, acetate, butyrate, succinate, propionate, formic acid, citrate, glucose, fructose, arabinoose, sucrose, ethanol, methanol, glycerol, and α-mannitol at a concentration of 2 g/L with 0.36 g/L (2 mM) of Na-glutamate as a nitrogen source. The concentration of 2 g/L of carbon was tested because it is a suitable concentration for the formation of total VFAs in hydrogen production by *Rhodobacter* sp. [13,43]. The capability of the KKU-PS1 strain to produce hydrogen using different nitrogen sources was examined. The experiments were conducted at a concentration of 2 mM of organic nitrogen (Na-glutamate) and inorganic nitrogen (ammonium sulfate) with a 2 g/L of dl-malic acid as the carbon source. Two micromolar of Na-glutamate was reported as the optimum nitrogen concentration for obtaining maximal hydrogen production by *R. sphaeroides* O.U.001 [12]. Therefore, a nitrogen concentration of 2 mM was used to test the capability of the KKU-PS1 strain in producing hydrogen. All of the experiments were performed in 60 mL serum bottles with a working volume of 40 mL. The fermentation broth contained 36 mL of sterile hydrogen production medium (HPM) and 10% (v/v) inoculum (cell concentrations of 10^6–10^7 cells/mL). The HPM consists of 2.0 g/L of dl-malic acid, 0.5 g/L of Na-glutamate, 3.9 g/L of KHPO₄, 2.8 g/L of KH₂PO₄, 0.2 g/L of MgSO₄ ×7 H₂O, 0.01 g/L of Na₂MO₄ ×2 H₂O, and 2 mL/L of a stock solution of 5 g/L FeSO₄ (Fe-EDTA complex). The initial pH of the HPM was adjusted to 7.0 using either NaOH pellets or 4 M HCl. Trace elements were added as described above. The HPM medium was sterilized at 121°C for 15 min before being used in hydrogen production. All of the above tests were done under anaerobic conditions. The bottles were closed with a rubber stopper and capped with an aluminum seal. Subsequently, the head space of the bottles was flushed with argon for 3 min. The serum bottles were incubated at 30°C, horizontally shaken at 150 rpm, and illuminated at an intensity of 2500 lx in an incubator shaker (WIS-10R, Wisd Laboratory Instruments, Korea) using LED lamps.

2.6. Optimization of initial pH and incubation temperature for bio-hydrogen production

2.6.1. Experimental design

RSM with CCD was applied to determine the major and interactive effects of two independent factors (Table 1), i.e., initial pH (6–8) and incubation temperature (22–38°C). The ranges of initial pH and temperature were chosen as the result of a literature search. Previous reports indicated that an initial pH of 7 was found to be optimal for both cell growth and hydrogen production in PNSB [10,44,45] while hydrogen cannot be produced at an initial pH of 5 [44,45]. Therefore, the initial pH tested in this study was in the range of 6–8. There were several temperature ranges for photo-hydrogen production by PNSB. For example, the temperature range hydrogen production by *Rhodobacter* sp. was between 31–36°C [31] and 27.5–32.5°C for *R. palustris* CQK 01 [17]. Thus, in order to cover the possible optimum temperature for hydrogen production by the KKU–PS1 strain, fermentation at 22–38°C was studied. Maximum cumulative hydrogen production (Hmax) and maximum hydrogen production rate (Rm) were selected as the desirable responses in a batch culture. The statistical analysis and test factors of X_i were coded as x_i values according to the following [Equation 1]

\[ x_i = (X_i - X_0) / \Delta X_i \]

[Equation 1]

where x_i is the coded value of the variable, X_i is the actual value of the independent variable, X_0 is the actual value of X_i at the center point and \( \Delta X_i \) is the step change value. A quadratic model [Equation 2] [46] was used to optimize the key environmental factors.

\[ Y_i = \beta_0 + \Sigma \beta_j X_j + \Sigma \beta_{ij} X_j^2 + \Sigma \beta_{ij} X_i X_j \]

[Equation 2]

where \( Y_i \) is the predicted response (Hmax or Rm), \( \beta_0 \) is a constant, \( \beta_j \) is the linear coefficient, \( \beta_{ij} \) is the squared coefficient, \( \beta_{ij} \) is the interaction coefficient, and x_i is the variable. The response variables (Hmax and Rm) were fitted using a predictive polynomial quadratic equation [Equation 2] in order to correlate the response variable to the independent variables [47,48]. The test conditions were designed with experimental data.

| Run | Initial pH (X_1) | Incubation temperature (X_2) | Hmax (mL H₂/L) | Rm (mL H₂/h) | Incubation time (d) |
|-----|-----------------|-------------------------------|--------------|--------------|-------------------|
| 1   | 0               | 7.0                           | 1128         | 5.1          | 12                |
| 2   | -1              | 6.5                           | 1166         | 4.6          | 14                |
| 3   | -2              | 6.0                           | 1106         | 3.1          | 19                |
| 4   | 2               | 8.0                           | 686          | 5.0          | 4.4               |
| 5   | 0               | 7.0                           | 1100         | 5.6          | 5.0               |
| 6   | -1              | 6.5                           | 977          | 3.5          | 14                |
| 7   | 1               | 7.5                           | 783          | 3.1          | 13                |
| 8   | 0               | 7.0                           | 1105         | 5.3          | 5.0               |
| 9   | 0               | 7.0                           | 1109         | 4.8          | 5.0               |
| 10  | 0               | 7.0                           | 182          | 1.9          | 7                 |
| 11  | 1               | 7.5                           | 990          | 4.4          | 5.4               |
| 12  | 0               | 7.0                           | 1170         | 5.1          | 5.0               |
| 13  | 0               | 7.0                           | 1338         | 5.4          | 5.0               |

Hmax: maximum cumulative hydrogen production; Rm: maximum hydrogen production rate.
Graphical analysis was performed using the statistical software, Design-Expert 7.0.0 Demo version (Stat-Ease, Inc., Minneapolis, MN, USA). A differentiation calculation was then used to predict the optimum values of the different factors simultaneously maximizing \( H_{\text{max}} \) and \( R_m \) of the new isolate in batch fermentation.

### 2.6.2. Bio-hydrogen production from malic acid by the new isolate

Seed inocula were grown on basal medium in 60 mL serum bottles with a working volume of 40 mL, incubated at 30°C, horizontally shaken at 150 rpm and illuminated at a controlled light intensity of 2500 lx. After 48 h of incubation, 40 mL of culture was transferred to 360 mL of basal medium in 600 mL serum bottles. After 24 h of subculture, the culture was used as the inoculum of a batch experiment. Malic acid at a concentration of 2 mM was used as the model substrate to produce hydrogen in this study. This is because malic acid can be easily used as an energy source for supporting hydrogen formation by directly entering the tricarboxylic acid (TCA) cycle [10]. Na-glutamate at a concentration of 3 mM (0.5 g/L) was used as the nitrogen source. The cultures (mid-log phase) were harvested by centrifugation at 7000 rpm for 10 min. An initial cell concentration of 0.52 g cell dry weight (cdw)/L and 10% (v/v) inoculum was used for hydrogen production. To assess hydrogen production, the experiment was carried out in 300 mL serum bottles with a working volume of 40 mL, incubated at 30°C, horizontally shaken at 150 rpm and illuminated at a controlled light intensity of 2500, 5000, 7500 and 10,000 lx on hydrogen production by the PNSB isolates were examined. All experiments were carried out under anaerobic conditions as described above.

### 2.7. Analytical methods

CDW was determined in four replicates using 7 mL culture samples. After filtration through a 0.45 μm membrane, the cells were washed with distilled water and dried at 85°C for 12 h (modified from Bianchi et al. [49]). The cell concentration was determined by measuring the absorbance at 660 nm with a UV–VIS Spectrophotometer (UVmini-1240, Shimadzu, Japan). An absorbance of 1.5 units was equivalent to 0.52 g cdw/L. The pH was measured using a digital pH meter (Sartorius, Germany).

The concentrations of VFAs (malic, acetic, butyric, propionic, lactic, formic, citric and succinic acids) in the liquid samples were analyzed using high performance liquid chromatography (HPLC) (Shimadzu LC-20AD, Shimadzu, Tokyo, Japan), employing refractive index (RI) and ultraviolet (UV) detectors with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). The oven temperature was 45°C. The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.5 mL/min [50].

The volume of the biogas was measured by releasing pressure from each of the serum bottles using wetted glass syringes ranging in size from 5 to 50 mL [51]. The resulting biogas was collected every 12 h or 24 h for the analysis of its composition and hydrogen content using a Shimadzu GC-2014 apparatus equipped with a 2 m stainless steel column packed with Shin carbon (50/80 mesh), following Fangkum and Reungsang [52]. Preparation of the liquid samples prior to HPLC analysis was performed according to Saraphirom and Reungsang [53].
2.8. Calculations

Hydrogen gas production was calculated from head-space measurements of hydrogen composition and the total volume of hydrogen produced using a mass balance after each time interval [54]. The hydrogen production rate (HPR) (mL H₂/L-h) was calculated by dividing \( H_{\text{max}} \) (mL H₂/L culture) by the incubation time (h). \( H_{\text{max}} \) was calculated using a modified Gompertz equation [55].

The \( \text{HY} \) (mol H₂/mol substrate) was calculated as the total amount of hydrogen (mol H₂) divided by the amount of substrate consumed (mol substrate) on a molar basis. The amount of hydrogen was determined using the ideal gas law [Equation 3], where \( P = \text{pressure (1 atm), } V = H_{\text{max}} \text{ (L H₂/L culture), } R = 0.0821 \text{ (L atm/K mol), and } T = \text{temperature (K)} [56].

\[
\text{Moles of hydrogen gas } (n) = \frac{PV}{RT}
\]  
[Equation 3]

The substrate conversion efficiency is a calculation of how much substrate has been converted for hydrogen production. The efficiency was determined by calculating the ratio of hydrogen produced to the amount theoretically possible from stoichiometric conversion of the substrate [Equation 4] [57].

\[
\text{Substrate conversion efficiency} = \frac{\text{mol } H_2 \text{ produced}}{\text{mol } H_2 \text{ theoretically possible}}
\]  
[Equation 4]

3. Results and discussion

3.1. Isolation of hydrogen-producing PNSB strains

Single red colonies isolated from the environmental samples were selected for further investigation. Red colonies indicated the presence of carotenoids and bacteriochlorophyll [44,58,59]. Most of the isolates were gram negative except those from CCS and FPW. These isolates were subsequently analyzed by PCR amplification to detect \( \text{nifD} \) and \( \text{nifH} \) genes before testing for hydrogen production ability.

The monoplex PCR targeting for each gene was individually performed to estimate the specificity of each primer. The primers for \( \text{nifD} \) and \( \text{nifH} \) yielded amplicons of 545 bp and 235 bp, respectively.

### Table 3

| Carbon source | \( \text{Rhodobacter sp.} \) KKU-PS1 | \( \text{R. sphaeroides} \) ZX-5 | \( \text{R. sphaeroides} \) KKU-PS1 | \( \text{R. sphaeroides} \) PS | \( \text{R. sulfidophilum} \) PS |
|---------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
|               | \( \text{H₂ production} \) | \( \text{OD}_{660 \text{nm}} \) | \( \text{H₂ production} \) | \( \text{OD}_{660 \text{nm}} \) | \( \text{H₂ production} \) | \( \text{H₂ production} \) |
| Organic acid  | Malate              | +   | 3.12 | +   | 2.29 | +   | ND   |
|               | Acetate             | +   | 4.28 | +   | 1.62 | +   | ND   |
|               | Butyrate            | +   | 4.65 | +   | 4.12 | +   | ND   |
|               | Succinate           | +   | 2.60 | +   | 2.44 | +   | 2.42 | +   |
|               | Lactate             | +   | 2.16 | +   | 2.42 | +   | 4.22 | +   |
|               | Propionate          | +   | 2.31 | +   | 2.45 | 2   | 2.42 | +   |
|               | Formic acid         | -   | 2.17 | ND  | ND   | ND  | ND   |
|               | Citrate             | -   | 0.22 | ND  | ND   | ND  | ND   |
| Carbohydrate  | Glucose             | +   | 3.64 | +   | 2.12 | +   | ND   |
|               | Fructose            | +   | 3.47 | +   | 2.41 | +   | ND   |
|               | Arabinose           | -   | 0.82 | +   | 2.58 | +   | ND   |
|               | Sucrose             | -   | 1.61 | +   | 2.05 | +   | ND   |
| Other         | \( \text{NaCO₃} \)  | +   | 1.54 | ND  | ND   | ND  | ND   |
|               | Ethanol             | -   | 0.94 | ND  | 0.75 | +   | -    |
|               | Methanol            | -   | 0.69 | ND  | ND   | ND  | ND   |
|               | Glycerol            | -   | 2.84 | ND  | ND   | ND  | ND   |
|               | D-mannitol          | +   | 4.38 | +   | 2.55 | +   | ND   |
| Reference     | This study          | [44] | [9]  | [70] |

Notes: + = presence of \( \text{H₂} \) production; - = absence of \( \text{H₂} \) production; ± = partially utilized and ND = not determined.
when DL-malic acid was used as a carbon source (Fig. 1 and Fig. 2). Only the nifD gene, they still could efficiently produce hydrogen. The nifH gene was not observed. Even though these isolates contained the nifD gene (Fig. 1, lanes 5, 6, 7, 8 and 9) while the control strain was contaminated (Fig. 1, lane 10). The results from PCR amplification indicated that only five isolates (ND1 and 2, NH1 and 2) was done to simultaneously detect both genes (nifD and nifH) (Fig. 1, lane 4). *R. palustris* CGA009 was used as a positive control (Fig. 1, lanes 2, 3 and 4) while sterile water was used as a negative control to confirm that no amplicons were observed (Fig. 1, lane 10). The results from PCR amplification indicated that only five isolates contained the nifD gene (Fig. 1, lanes 5, 6, 7, 8 and 9) while the nifH gene was not observed. Even though these five isolates contained only the nifD gene, they still could efficiently produce hydrogen when DL-malic acid was used as a carbon source (Fig. 1 and Fig. 2). Theoretically, Mo nitrogenase activity involves a synergy between the Fe protein (*NifH*) and the MoFe protein (*NifDK*) ([Danyal et al.](#60)); [Seefeldt et al.](#61); [Hu and Ribbe](#24)). The MoFe protein is an αβ2 heterotetramer composed of two types of metal clusters ([62]) that aggregate into large proteins enabling easy detection of nifD. This site specific for electron transfer activity and responsible for gene expression ([61]).

Among the five isolates, the strain isolated from the methane fermentation broths of an UASB reactor showed the highest *H*<sub>max</sub>, HY and *R*<sub>m</sub> of 881 mL H<sub>2</sub>/L, 2.42 mol H<sub>2</sub>/mol malate and 4.3 mL H<sub>2</sub>/L-h, respectively (Fig. 2 and Table 2). Thus, the UASB strain was further investigated for its hydrogen production capability. The possible reasons that the UASB strain exhibited the highest bio-hydrogen production potential might be due to its growth in a methane fermentation broth containing high concentrations of VFAs. The feedstock used to produce methane in the UASB was an acidic effluent (mainly butyric acid and propionic acid [data not shown]) coming from a sugarcane juice hydrogen fermentation process. Therefore, the UASB strain might have been able to adjust to the use of malic acid to produce hydrogen to a greater degree than isolates obtained from other environmental sources. Moreover, the difference in hydrogen production capability of the five isolates might have been due to the activities of the nitrogenase enzyme of each isolate. This speculation is supported by the report of Xie et al. ([63]) who found that nitrogenase activity had a positive correlation to hydrogen production.

### 3.2. Characterization of the UASB strain

The UASB strain was gram-negative, with ovoid red colonies and dimensions of 0.88–1.21 × 0.58–0.62 μm. Polar flagella were not observed. Cells were divided by binary fission. It could produce slime depending on the carbon source used and formed chains of cells (Fig. 3). Cultures were yellowish brown under anaerobic conditions and reddish brown in the presence of air. The absorption maxima of the UASB strain occurred at 471 and 505 nm due to the presence of carotenoids, and at 802 and 853 nm due to the presence of bacteriochlorophyll a ([44,64]). The partial 16S rRNA gene sequence (1300 bp) of the UASB strain was deposited in GenBank with the Accession No. KC478552. The 16S rRNA gene analysis demonstrated that the UASB strain showed high similarity (99%) to *R. sphaeroides*. Therefore, this strain was named *Rhodobacter* sp. KU-KPS1. A phylogenetic tree (Fig. 4) revealed that KU-KPS1 strain was closely related to strains JA460, JA252 and NMBL-01. The KU-KPS1 strain showed the ability to grow on a wide variety of carbon sources (Table 3). The phenotypic characteristics of the KU-KPS1 strain were consistent with those of *R. sphaeroides* KU-KPS except that the KU-KPS5 strain could not utilize propionate although it could grow on arabinose (Table 3). Based on the range of carbon sources used, the KU-KPS strain can be considered a new strain within the species of *R. sphaeroides*.

### 3.3. Effect of carbon sources on bio-hydrogen production

KU-KPS1 was able to use all of the carbon sources tested for cell growth and was able to use ten of carbon sources tested for producing hydrogen (Table 3). This suggested that the KU-KPS1 strain had the potential to produce hydrogen and cells using VFAs and simple sugars as carbon sources. The highest cumulative hydrogen production of 881 mL H<sub>2</sub>/L was achieved when malic acid was used as the carbon source (Table 2, Fig. 2), while maximum cell growth was obtained when butyrate was used as the carbon source (Table 3). The carbon source is usually the most important factor affecting the metabolism of photo-hydrogen production and cell synthesis ([65,66]). The majority of carbon sources are utilized for cell synthesis. Only a few substrates can be used for photo-hydrogen production under suitable conditions ([26]). Differences in hydrogen production utilizing different carbon sources were observed ([67]). The discrepancy might be due to the variations in the electron transfer capabilities of cofactor compounds required for nitrogenase activity. The metabolic pathways of PNSB are different as well ([26]).

![Fig. 5. Response surface plots showing the effect of initial pH and incubation temperature on *H*<sub>max</sub> (a) and *R*<sub>m</sub> (b) from maltic acid by KRU-KPS1.](image)
Table 5
Confirmation photo-hydrogen production experiment.

| Run | Condition | Initial pH | Temperature (°C) | Hmax (mL H₂/L) | Rm (mL H₂/L·h) | HY (mol H₂/mol malate) |
|-----|-----------|------------|-----------------|---------------|----------------|------------------------|
| 1   | Center    | 7.0        | 30              | 118           | 1124           | 4.6                    |
| 2   | Worst     | 6.5        | 26.0            | 1209          | 1299           | 5.3                    |
| 3   | Height    | 7.5        | 34.0            | 838           | 645            | 4.1                    |

\( H_{\text{max}} \): maximum cumulative hydrogen production; \( R_{\text{m}} \): maximum hydrogen production rate; \( Y_{\text{HY}} \): hydrogen yield.

3.4. Effect of nitrogen source on bio-hydrogen production

The KKU-PS1 strain produced large amounts of hydrogen with an organic nitrogen source, i.e., Na-glutamate (881 mL H₂/L). With 2 mM of inorganic nitrogen, lower amounts of hydrogen were produced than with the same concentration of inorganic nitrogen, i.e., 530 mL H₂/L of NH₄Cl. This is due to direct solubilization of organic nitrogen into proteins or transfer into other nitrogenous cellular components [68]. Microorganisms require more developed metabolism to use inorganic nitrogen for amino acid and protein production [67]. Additionally, a higher ammonium ion concentration metabolism to use inorganic nitrogen for amino acid and protein production [69].

Table 6
Comparison of hydrogen production by different microorganisms.

| Microorganism     | Malate con. (mM) | Conditions | Hmax (mL H₂/L) | Substrate conversion efficiency (%) | Substrate degradation (%) | Incubation time (d) | Reference |
|-------------------|------------------|------------|---------------|--------------------------------------|--------------------------|---------------------|-----------|
|                   |                  | pH | Temp. (°C)   | Illumination intensity              |                          |                     |           |
| R. sphaeroides KD131 | 30               | 7.0 | 30           | 6831 lx*                           | 1190                     | 22.2                | 93.1      | 3 [10]    |
| R. sphaeroides NMML-01 | 30             | 7.0 | 32 ± 2       | 1800 lx                             | 2275                     | 68.3                | ND        | 15 [73]   |
| R. sphaeroides RV | 45               | 7.0 | 32           | 4000 lx                            | 284                      | 7.0                 | 93.4      | 4 [74]    |
| R. sphaeroides ZK-5 | 30               | 7.0 | 30 ± 1       | 5000 lx                            | 3157                     | 71.25               | ND        | 3 [44]    |
| R. sphaeroides O.U.001 | 7.5          | 6.8 ± 0.2 | 32 ± 2       | 932 lx*                            | 650                      | 44.9                | ND        | 5 [75]    |
| Rhodobacter sp. KKU-PS1 | 15            | 7.0 | 25.6         | 7500 lx                            | 1339                     | 64.7                | 100       | 8 This study |

\( \text{ND} \) = not determined; \( \text{H}_{\text{max}} \): maximum cumulative hydrogen production.

* = Illumination intensity was calculated (1 lx = 0.0161028 W/m²) [83].
5.4 mL H₂/L-h, respectively. These values were different from the observed values by only 6.5% and 21%, respectively. Hence, RSM with CCD was a useful tool to optimize photo-hydrogen production from DL-malic acid by KKU-PS1. The results under the optimum conditions in this study were compared to results from other experiments using DL-malic acid as a carbon source for hydrogen production (Table 6). The H_max value obtained by the KKU-PS1 strain was comparable to that observed in R. sphaeroides KD131 [10], and greater than the levels found in R. sphaeroides RV [74] and R. sphaeroides O.U.001 [75]. However, the H_max value of KKU-PS1 was lower than that obtained using R. sphaeroides NMBL-01 [73] and R. sphaeroides ZX-5 [44]. The KKU-PS1 strain could completely utilize DL-malic acid. This can be considered its distinguishing characteristic. The maximum malic acid intensity from 2500 to 10,000 lx shortened the lag time (Table 7).

### Table 7

| Light (lx) | H_max (mL H2/L) | R_m (mL H2/L-h) | λ (h) | R² | HY (mol H2/molmalate) | Incubation time (d) |
|-----------|----------------|----------------|-------|----|----------------------|---------------------|
| 2500      | 1353 ± 34      | 6.8 ± 0.4      | 57.1 ± 5.6 | 0.99 | 3.92 | 12 |
| 5000      | 1382 ± 38      | 8.1 ± 0.6      | 47.9 ± 5.6 | 0.99 | 3.95 | 10 |
| 7500      | 1339 ± 24      | 12.0 ± 0.8     | 40.9 ± 3.8 | 0.99 | 3.88 | 8  |
| 10,000    | 1258 ± 16      | 13.3 ± 0.9     | 39.8 ± 2.8 | 0.99 | 3.67 | 8  |

H_max: maximum cumulative hydrogen production; R_m: maximum hydrogen production rate; λ: lag phase time; R²: the determination coefficient; HY: hydrogen yield.

From these results, an optimum illumination intensity of 7500 lx gave the best conditions for the KKU-PS1 strain. Under these conditions, maximal values of H_max, HY, and R_m of 1339 mL H2/L, 3.88 mol H2/molmalate, and 12.0 mL H2/L-h were attained, respectively. Simultaneously, the shortest lag times of 40.9 h and 8 d resulted. The increase in H_max and R_m as light intensity increased from 2500 to 5000 lx which occurred because at a high illumination intensity, more ATP and reducing power were supplied to the photosynthetic system. These are essential for photo-hydrogen producing bacteria [77,78]. Large amounts of ATP (in the form of light energy) are required for nitrogenase activity to produce hydrogen and synthesize the cells. However, the results indicated that higher illumination intensity became a limiting factor for hydrogen production. This suggested that hydrogen production by the KKU-PS1 strain was saturated at an illumination intensity of 7500 lx. Light saturation might have occurred when the photosynthetic system provided excess ATP and Fd_red compared to the capacity of nitrogenase enzyme [79]. Photo inhibition was observed in the studies of Kim et al. [80] who found that the light saturation occurred when light intensity was higher than 200 W/m² while Cai and Wang [70] reported that a light intensity of 6000 lx became an inhibiting factor for hydrogen production in *Rhodovulum sulfidophilum* P5.

In this study, LED were used as the light source because they have a suitable and specific wavelength range (770–920 nm) for bacteriochlorophyll a [81]. Moreover, LED light sources have several additional benefits, including lower energy consumption, lower heat generation, longer life expectancy and improved performance regarding photo-hydrogen production [27]. Other types of light sources used previous studies of photo-fermentation of hydrogen including halogen [65,80] and tungsten lamps [13,44,82].

### 3.7. Effect of illumination intensity on bio-hydrogen production

The results show that illumination intensity plays an important role in photo-hydrogen production by the KKU-PS1 strain. Its R_m increased with increasing illumination intensity. The maximum H_max was obtained at 10,000 lx. H_max slightly increased when the light intensity was increased from 2500 to 5000 lx and then decreased with further increases in light intensity. Additionally, an increase in light intensity from 2500 to 10,000 lx shortened the lag time (Table 7).

### 3.8. Photo hydrogen production under the optimum conditions

Photo-hydrogen production of the KKU-PS1 strain in batch fermentation under the optimum conditions (initial pH 7.0, 25.6°C and 7500 lx) was investigated using DL-malic acid as the carbon source.
4. Concluding remarks

Rhodobacter sp. KKU-PS1 was isolated from the methanation fermentation broth of an UASB reactor. The KKU-PS1 strain efficiently utilized ten carbon sources for hydrogen production. Na-glutamate was a preferred nitrogen source while malic acid was a preferred carbon source for hydrogen production. Initial pH and incubation temperature had a significant effect (p ≤ 0.05) on Hmax and Rm, but there were no interaction effects between the initial pH and incubation temperature. Simultaneous maximization of Hmax and Rm occurred at the optimal initial pH of 7.0, an incubation temperature of 25.6°C and a light intensity of 2500 lx. Under these conditions, maximum Hmax and Rm values of 1353 mL H2/L and 6.8 mL H2/L/h were obtained, respectively. Further investigations on the light intensity using the optimal initial pH and incubation temperature indicated that a light intensity of 7500 lx increased Rm by approximately two fold. Additionally, an increase in illumination intensity shortened the lag phase for hydrogen production.

Conflict of interest statement

We have declared no conflict of interest.

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