bio-protocol

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H_2 Production from Methyl Viologen–Dependent Hydrogenase Activity Monitored by Gas Chromatography

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Abstract

Bio-hydrogen production is an eco-friendly alternative to commercial H_2 production, taking advantage of natural systems. Microbial hydrogenases play a main role in biological mechanisms, catalyzing proton reduction to molecular hydrogen (H_2) formation under ambient conditions. Direct determination is an important approach to screen bacteria with active hydrogenase and accurately quantify the amount of H_2 production. Here, we present a detailed protocol for determining hydrogenase activity based on H_2 production using methyl viologen (MV^{2+}) as an artificial reductant, directly monitored by gas chromatography. Recombinant *Escherichia coli* is used as a hydrogenase-enriched model in this study. Even so, this protocol can be applied to determine hydrogenase activity in all biological samples.

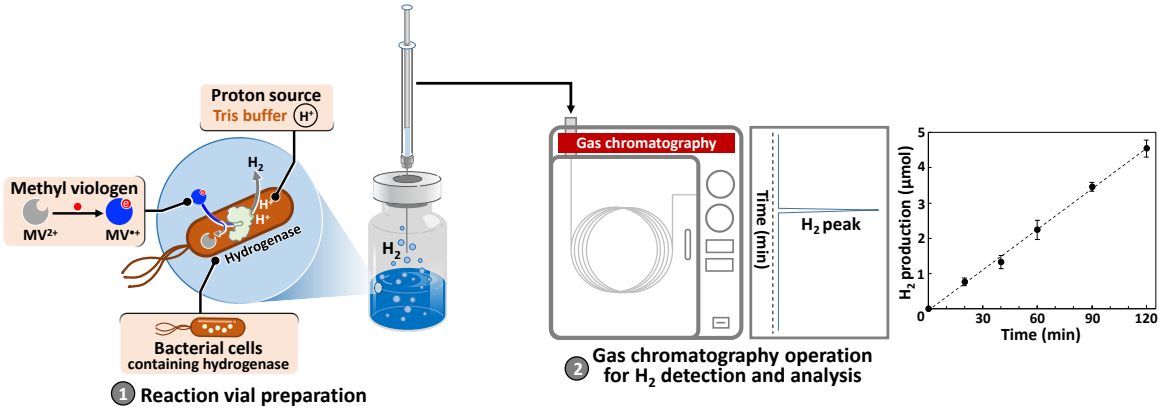
Key features

- This protocol is optimized for a wide variety of biological samples; both purified hydrogenase (in vitro) and intracellular hydrogenase (in vivo) systems.
- Direct, quantitative, and accurate method to detect the amount of H_2 by gas chromatography with reproducibility.
- Requires only 2 h to complete and allows testing various conditions simultaneously.
- Kinetic plot of H_2 production allows to analyze kinetic parameters and estimate the efficiency of hydrogenase from different organisms.

Keywords: Hydrogenase, Hydrogen, Methyl viologen, Gas chromatography, Biocatalyst

This protocol is used in: Appl. Catal. A: Gen. (2023), DOI: 10.1016/j.apcata.2022.119019

Graphical overview



Background

Although hydrogen (H_2) has been considered as a clean energy carrier, most of today's commercial H_2 production is from fossil fuels, which emit greenhouse gases into the atmosphere. Finding a more sustainable alternative remains a challenge. It is well known that, for over a million years of evolution, microorganisms have acted as a natural H_2 production plant through the action of enzymes, namely the hydrogenase family (Tard and Pickett, 2009). With efficient biochemical mechanisms, it is possible to develop an environmentally friendly process for H_2 production at ambient conditions by taking advantage of hydrogenase-expressing microorganisms. Among the enzymes with different catalytic sites, [FeFe]-hydrogenase is more efficient than [NiFe]- and [Fe]-types in H_2 production with NADH as a reductant in a natural mechanism, as shown in Figure S1 in the Supporting materials (Ogo et al., 2020; Xuan et al., 2023). To screen microorganisms carrying active hydrogenase in a laboratory scale, methyl viologen (MV^{2+}), which is compatible with many enzymes (Orgill et al., 2015), can also be used as an artificial reductant in this reaction. According to the theory, a more negative redox potential of methyl viologen [$E(MV^{2+}/MV^{\bullet+})$ = -0.446 V vs. normal hydrogen electrode (NHE)] provides a favorable potential scale for proton reduction [$E(H^+/H_2)$ = -0.41 V vs. NHE] at physiological pH.

In this protocol, a reduced methyl viologen ($MV^{\bullet+}$) is formed in sodium dithionite ($Na_2S_2O_4$) solution to serve as an artificial chemical reductant. With cell permeability, $MV^{\bullet+}$ can penetrate and transfer electrons to intracellular hydrogenase (Kosem et al., 2023), as shown in Figure 1.

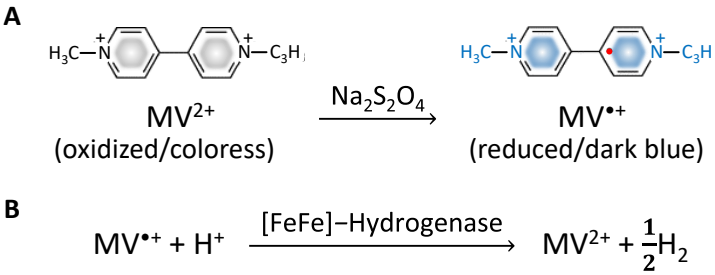


Figure 1. Methyl viologen (MV^{2+}) as an artificial reductant in hydrogenase activity assay. (A) MV^{2+} reduction to $MV^{\bullet+}$ formation in $Na_2S_2O_4$ solution. (B) H_2 production from the $MV^{\bullet+}$-dependent reaction of [FeFe]-hydrogenase.

The amount of H_2 generated is directly monitored by gas chromatography. To avoid the detrimental effect of atmospheric oxygen and enhance biocatalytic activity, the reactions are carried out under anaerobic conditions at 37 °C. This method can be applied to determine H_2 production capacity of various biological samples such as whole cells, crude cell extracts, or purified enzymes.

Materials and reagents

Biological materials

1. Hydrogenase-expressing *Escherichia coli* carrying *hydA*, *hydE*, *hydF*, and *hydG* genes (this recombinant bacterial strain was constructed in our laboratory according to our previous report in Kosem et al., 2023).
   Note: The function of each protein expressed from specific genes in the processes of hydrogenase maturation was reported in many published articles, such as Broderick et al. (2014) and Lubitz et al. (2014).

Reagents

1. LB broth, Miller (Nacalai Tesque, catalog number: 20068-75)
2. Methyl viologen (Tokyo Chemical Industry, catalog number: D3685)
3. Sodium dithionite ($Na_2S_2O_4$) (Sigma-Aldrich, catalog number: 71699-250G)
4. Pierce™ BCA Protein Assay kit (Thermo Scientific, catalog number: 23227)
5. Sodium chloride (NaCl) (Wako Chemical, catalog number: 191-01665)
6. Tris (hydroxymethyl) aminomethane (Tris) (Nacalai Tesque, catalog number: 35434-21)
7. Hydrochloric acid (HCl) (Wako Chemical, catalog number: 080-0106)
8. Trichloroacetic acid (TCA) (Nacalai Tesque, catalog number: 34637-14)
9. 3-(N-morpholino)propanesulfonic acid (MOPS) (Nacalai Tesque, catalog number: 23415-25)
10. Sodium hydroxide (NaOH) (Chameleon Reagent, catalog number: 000-75165)
11. Ampicillin sodium (Wako, catalog number: 014-23302)
12. Streptomycin sulfate (Wako, catalog number: 194-08512)
13. Glucose (Nacalai Tesque, catalog number: 16805-35)
14. Ferric ammonium citrate (Nacalai Tesque, catalog number: 19425-12)
15. L-cysteine (Nacalai Tesque, catalog number: 10309-12)
16. Sodium fumarate (TCI, catalog number: F0070)
17. Isopropyl-β-D-thiogalactopyranoside (IPTG) (FujiFilm, catalog number: 094-05144)

Solutions

1. LB (Luria-Bertani) broth (see Recipes)
2. 1 M MOPS-NaOH (see Recipes)
3. 100 mg/mL Ampicillin sodium (see Recipes)
4. 40 mg/mL Streptomycin sulfate (see Recipes)
5. 50% (w/v) Glucose (see Recipes)
6. 250 mg/mL Ferric ammonium citrate (see Recipes)
7. 1 M Sodium fumarate (see Recipes)
8. 1 M IPTG (see Recipes)
9. 0.9% (w/v) NaCl (see Recipes)
10. 50 mM MV^{2+}/$Na_2S_2O_4$ solution (see Recipes)
11. 1 M Tris-HCl pH 7 (see Recipes)

Recipes

1. **LB broth**
   Note: Dissolve the medium and all reagents in a 500 mL Erlenmeyer flask and then autoclave at 121 °C for 15 min before use.

Reagent	Final concentration	Quantity
LB broth	2.5 % (w/v)	5 g

1 M MOPS-NaOH (pH 7.4) (Recipe 2)	100 mM	20 mL
Water	n/a	180 mL
Total	n/a	200 mL
The following (see Recipes 3–8) are added after autoclaving:		
100 mg/mL Ampicillin sodium	100 µg/mL	0.2 mL
40 mg/mL Streptomycin sulfate	40 µg/mL	0.2 mL
50% (w/v) Glucose	0.5% (w/v)	2 mL
250 mg/mL Ferric ammonium citrate	250 µg/mL	0.2 mL
L-cysteine	2 mM	50 mg
1 M Sodium fumarate	20 mM	4 mL
1 M IPTG	1 mM	0.2 mL

2. **1 M MOPS-NaOH pH 7.4**

Note: Dissolve in a 100 mL beaker on a magnetic stirrer.

Reagent	Final concentration	Quantity
MOPS	1 M	20.9 g
Water	n/a	80 mL
NaOH (8 M)	n/a	Slowly add until pH 7.4
Total	n/a	Make up final volume to 100 mL

3. **100 mg/mL Ampicillin sodium**

Note: Dissolve in a 5 mL microcentrifuge tube and mix thoroughly with gentle shaking by hand. Aliquot the stock solution in 1 mL microcentrifuge tubes (500 µL per tube) and store at -20 °C until use.

Reagent	Final concentration	Quantity
Ampicillin sodium	100 mg/mL	0.5 g
Sterilized water	n/a	5 mL
Total	n/a	5 mL

4. **40 mg/mL Streptomycin sulfate**

Note: Dissolve in a 5 mL microcentrifuge tube and mix thoroughly with gentle shaking by hand. Aliquot the stock solution in 1 mL microcentrifuge tubes (500 µL per tube) and store at -20 °C until use.

Reagent	Final concentration	Quantity
Streptomycin sulfate	40 mg/mL	0.2 g
Sterilized water	n/a	5 mL
Total	n/a	5 mL

5. **50% (w/v) Glucose**

Note: Dissolve glucose powder with warm sterilized water in a 100 mL beaker on a magnetic stirrer. Once it has completely dissolved, bring the volume up to 100 mL total. Aliquot the stock solution in 15 mL tubes (10 mL per tube) and store at -20 °C until use.

Reagent	Final concentration	Quantity
Glucose	50% (w/v)	50 g
Sterilized water	n/a	60 mL
Total	n/a	Make up final volume to 100 mL

6. **250 mg/mL Ferric ammonium citrate**

Note: Dissolve in a 5 mL microcentrifuge tube and mix thoroughly with gentle shaking by hand. Aliquot the stock solution in 1 mL microcentrifuge tubes (500 µL per tube) and store at -20 °C until use.

Reagent	Final concentration	Quantity
Ferric ammonium citrate	250 mg/mL	1.25 g
Sterilized water	n/a	5 mL

Total	n/a	5 mL

7. 1 M Sodium fumarate

Note: Dissolve in a 50 mL tube and mix thoroughly with gentle shaking by hand. Aliquot the stock solution in 15 mL tubes (10 mL per tube) and store at -20 °C until use.

Reagent	Final concentration	Quantity
Sodium fumarate	1 M	8.0 g
Sterilized water	n/a	50 mL
Total	n/a	50 mL

8. 1 M IPTG

Note: Dissolve in a 5 mL microcentrifuge tube and mix thoroughly with gentle shaking by hand. Aliquot the stock solution in 1 mL microcentrifuge tubes (500 µL per tube) and store at -20 °C until use.

Reagent	Final concentration	Quantity
IPTG	1 M	1.2 g
Sterilized water	n/a	5 mL
Total	n/a	5 mL

9. 0.9% (w/v) NaCl solution

Note: Dissolve in a 100 mL beaker on a magnetic stirrer.

Reagent	Final concentration	Quantity
NaCl	0.9% (w/v)	0.9 g
Water	n/a	100 mL
Total	n/a	100 mL

10. 50 mM MV^{2+}/$Na_2S_2O_4$ solution

Note: Prepare fresh in an anaerobic sealed vial inside a glove box and mix thoroughly with gentle shaking by hand.

Reagent	Final concentration	Quantity
Methyl viologen	50 mM	0.0257 g
$Na_2S_2O_4$	250 mM	0.0870 g
Water	n/a	2 mL
Total	n/a	2 mL

11. 1 M Tris-HCl pH 7

Note: Dissolve in a 100 mL beaker on a magnetic stirrer. Adjust pH with 3 M HCl in a fume hood and strictly following the Kyushu University Guidelines for Safety in Education: Laboratory Activities. A safety data sheet from the chemical company as shown in Guideline 1 in the Supporting materials.

Reagent	Final concentration	Quantity
Tris	1 M	12.1 g
Water	n/a	80 mL
HCl (3 M)	n/a	Slowly add until pH 7
Total	n/a	Make up final volume to 100 mL

Laboratory supplies

1. Glass vial No. 3, 10 mL size (ASONE, catalog number: 5-111-03)
2. Rubber stopper (ASONE, catalog number: 5-112-01)
3. Aluminum cap (ASONE, catalog number: 5-112-03)
4. 1 mL Syringe (Terumo, catalog number: SS-01T)
5. Needle No. 27 G × 3/4" (Terumo, catalog number: NN-2719S)

6. 10 µL pipette tip (Molecular BioProducts, catalog number: 3510-05)
7. 200 µL pipette tip (Violamo, catalog number: 3-6504-12)
8. 1,000 µL pipette tip (Violamo, catalog number: 3-6504-13)
9. 1 mL microcentrifuge tube (Quality Scientific Plastics, catalog number: L-510-GRD-Q)
10. 5 mL microcentrifuge tube (ASONE, model: AST0500)
11. 96-well plate (ASONE, catalog number: 2-8085-02)
12. 99.999% N_2 gas (Air Liquide, catalog number: JAGA 13038)
13. Gloves (Showaglove, catalog number: 882.L.BLUE)
14. Paper towels
15. Disposable cuvettes (Violamo, model: UVC-Z8.5)

Equipment

1. 2–20 µL autoclavable micropipette (Nichipet Ex II, Nichiryo, catalog number: J16Y01961)
2. 20–200 µL autoclavable micropipette (Nichipet Ex II, Nichiryo, catalog number: J16Z00871)
3. 100–1,000 µL autoclavable micropipette (Nichipet Ex II, Nichiryo, catalog number: J16X11751)
4. accu-jet® pro pipette controller (BRAND®, catalog number: Z671533)
5. High-speed micro centrifuge (Hitachi, model: CF16RN)
6. Personal centrifuge (Front Lab, model: FLD2012)
7. Gas chromatography (Shimadzu Corp., model: GC-8A)
8. Thermal conductivity detector (Shimadzu Corp., model: GC-8AIT)
9. Integrator C-R6A chromatopac (Shimadzu Corp., catalog number: 223-04500-38)
10. Molecular sieve 5A beads (GL Sciences Inc., catalog number: 1001-11503)
11. Stainless steel column, 2 m length × 3 mm diameter (Shimadzu Corp., catalog number: 201-48705-20)
12. Shaking water bath (Yamato Scientific, model: BW101)
13. Shaking incubator (EYELA, model: FYC-100)
14. Anaerobic glove box (MIWA, model: 1ADB-3)
15. Clean bench (Hitachi Appliances Inc., model: CCV-1300E)
16. Microplate reader (Corona Electric, model: SH-1000)
17. 500 mL Erlenmeyer flask with baffle (IWAKI, model: CTE33)
18. 250 mL centrifuge bottle (Nalgene, catalog number: B1033)
19. 20 mm vial crimper (Chromatography Research Supplies, catalog number: 320990)
20. 20 mm vial decapper pliers (Kebby, catalog number: D-20)
21. 1 mL gas tight syringe (ITO Corporation, catalog number: MS-GAN100)
22. Weighing balance with 0.0001 g accuracy (Metter Toledo, model: XS204)
23. Vortex mixer (Scientific Industries, model: SI-0286)
24. pH meter (Horiba Scientific, model: 9625)
25. Water distillation apparatus (Advantec, model: RFD240NA)
26. Magnetic stirrer (Pasorina Stirrer, model: CT-MINI)
27. 100 mL Beaker (AGC Iwaki, model: CTE33)
28. Fume hood (Oriental, model: TNV-STZ-1800HCS)

Software and datasets

1. Microsoft Excel
2. SF6 (version 5.6.0, Copyright© 2005 Corona Electric) for spectrophotometer

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Procedure

A. Cultivation of hydrogenase-expressing bacteria

1. In this protocol, a recombinant *E. coli* encoding *hydA*, *hydE*, *hydF,* and *hydG* genes for hydrogenase expression was utilized as a H_2-producing model constructed in our laboratory as reported in Honda et al. (2016) and Kosem et al. (2023).
   Note: This protocol can be applied to determine the efficiency of other hydrogenase-producing bacteria, as reported in Benoit et al. (2020) and Kosem et al. (2024).
2. Aerobically pre-culture the recombinant *E. coli* in a 500 mL Erlenmeyer flask with 200 mL of LB broth supplemented with 100 µg/mL of ampicillin sodium, 40 µg/mL of streptomycin sulfate, 0.5% (w/v) of glucose, 250 µg/mL of ferric ammonium citrate, and 100 mM MOPS/NaOH pH 7.4. Incubate the pre-culture flask at 37 °C placed on a shaking incubator at 120 rpm until the cell density reaches an optical density of 0.4 at OD_{600}, monitored by a spectrophotometer using a 1 mL cell suspension in a disposable cuvette. Then, transfer the 200 mL pre-cultured *E. coli* suspension into a 250 mL centrifuge bottle inside an anaerobic glove box supplemented with 2 mM L-cysteine (50 mg), 20 mM sodium fumarate (4 mL of 1 M stock solution), and 1 mM IPTG (0.2 mL of 1 M stock solution), and further incubate for 18 h in the glove box for [FeFe]-hydrogenase expression (Figure 2A).

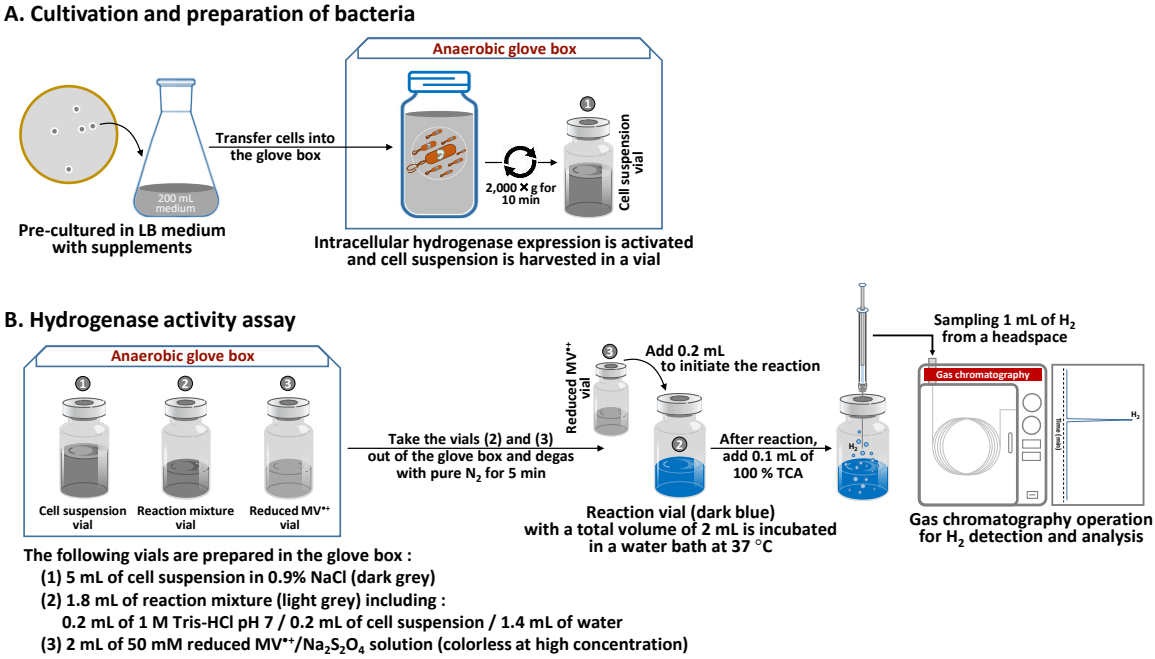

Figure 2. Experimental process of MV^{2+}-dependent hydrogenase activity

B. Cell harvesting and preparation

1. In the glove box, transfer 200 mL of cell suspension cultivated in LB medium from the Erlenmeyer flask to a centrifuge bottle with a silicone cap to preserve an anaerobic environment.
2. Centrifuge the bottle at 2,000× *g* for 10 min, discard the supernatant, and collect cell pellet.
3. Wash the cell pellet once with 5 mL of 0.9% NaCl solution and centrifuge at 2,000× *g* for 10 min.
4. To prepare cell suspension for experiments, resuspend the washed cell pellet in 5 mL of 0.9% NaCl solution (Figure 2A).
5. To standardize the protocol, the concentration of cells used in the reaction is based on total protein contents

measured by Pierce™ BCA Protein Assay kit in a 96-well plate.
Note: The protocol can be modified when different bacterial strains or biological samples are used.

C. Hydrogenase activity assay

1. Degas all chemical reagents with pure N_2 for 5 min and place them in the anaerobic glove box before use.
2. Prepare the following reagents as shown in Table 1 and Figure 2B.

Table 1. Reaction mixture preparation of $MV^{\bullet+}$-hydrogenase activity assay

Reagents	Applied volume	Final concentration
1 M Tris-HCl pH 7	0.2 mL	100 mM
10 mg/mL protein of cell suspension or extract	0.2 mL	1 mg/mL
Sterile deionized water	1.4 mL	
50 mM MV^{2+} in 250 mM $Na_2S_2O_4$ (reduced $MV^{\bullet+}$ solution)	0.2 mL	5 mM
Total	2 mL	

Notes:
a. The reaction mixture is prepared in a 10 mL glass vial and sealed with a rubber stopper and aluminum cap inside the glove box.
b. The protein concentration can be adjusted in different samples.
c. In case of a negative control, the same volume of sterile water is added instead of cell suspension or extracts.
d. The reduced $MV^{\bullet+}$ solution is prepared in a separate vial and sealed with a rubber stopper and aluminum cap inside the glove box.

3. Take the reaction vial and the $MV^{\bullet+}$ vial out of the glove box.
4. Purge the contaminated gases in each vial with pure N_2 for 5 min.
5. Initiate the reaction by adding 0.2 mL of 50 mM $MV^{2+}/Na_2S_2O_4$ solution into the reaction vial using a 1 mL syringe with needle No. 27 G × 3/4" and further incubate in a shaking water bath at 100 rpm and 37 °C.
Note: The reaction mixture turns dark blue after adding $MV^{2+}/Na_2S_2O_4$ solution into the reaction vial.
6. Every 20 min after incubation, terminate the reaction vial by adding 0.1 mL of 100% TCA (concentration of the commercial stock solution) using a 1 mL syringe with needle No. 27 G × 3/4".
7. Sample 1 mL of H_2 produced in a headspace of the reaction vial using a 1 mL gas tight syringe and vertically inject into a gas chromatograph for analysis. Here, a GC-8A gas chromatograph equipped with a thermal conductive detector and an integrator C-R6A chromatopac was used. The produced gas goes through molecular sieve 5A beads packed in a stainless steel column (2 m length × 3 mm diameter) with a carrier gas of argon. Operating parameters are shown in Table 2.

Table 2. Chromatographic operating parameters

Parameters	Units
Pressure of carrier gas, Ar	100 kPa
Injection volume	1 mL
Detector temperature	50 °C
Injection temperature	50 °C
Column temperature	50 °C
Detector current	60 mA

Data analysis

1. Quantification of H_2:
The accurate amount of H_2 produced from the reaction is calculated from a calibration curve between H_2

concentration vs. peak area obtained from the chromatogram of GC analysis (Figure 3).

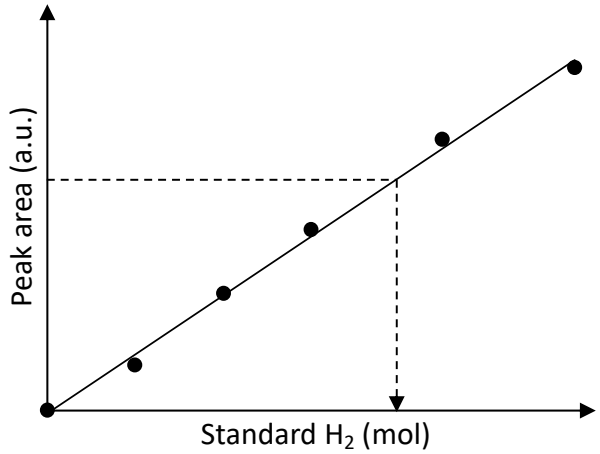

Figure 3. Calibration plot of standard H_2 vs. peak area by gas chromatography. According to the calibration curve, the amount of H_2 can be calculated from the following equation: H_2 (mol) = (Peak area – Intercept)/Slope.

2. Calculation of hydrogenase activity

$$\text{Hydrogenase activity (μmol/min/mg protein)} = \frac{Amount\ of\ H_2\ produced/Time}{C_{protein} \times V_{reaction}} \quad (1)$$

In which the amount of H_2 (μmol) in the headspace (Figure 4A) is measured at each time point and plotted to obtain the H_2 production rate (Figure 4B).
Time (minutes or hours) is the incubation time of the reaction.
Note: Normally it takes 1–2 h for the incubation period. The amount of H_2 production rate is presented in μmol per min.
$C_{protein}$ (mg/mL) is the final concentration of protein in the reaction mixture.
$V_{reaction}$ (mL) is the total volume of the reaction mixture.

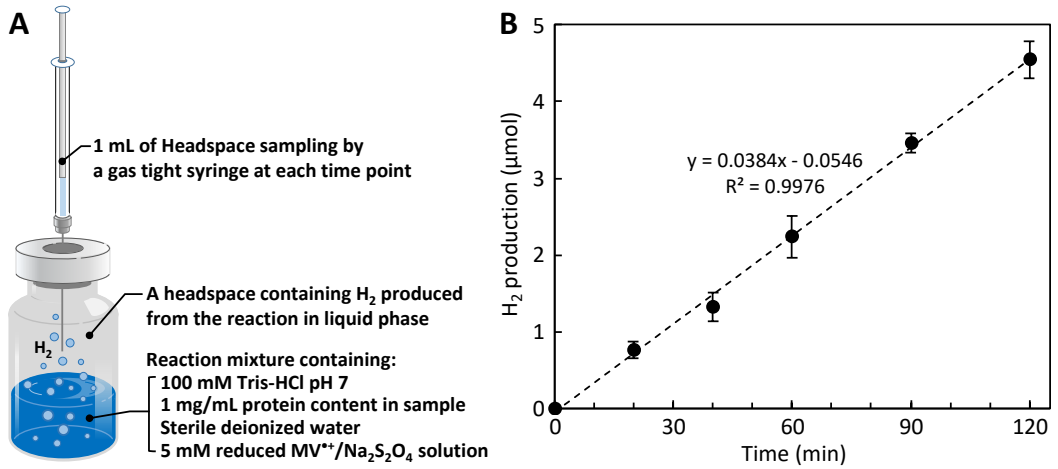

Figure 4. Methyl viologen ($MV^{\bullet+}$)-dependent hydrogenase activity assay. (A) Reaction vial containing the liquid phase of reaction mixture and the gas phase of H_2 produced in a headspace. (B) H_2 production rate is calculated from

a kinetic plot between the amount of H_2 in the y-axis vs. time in the x-axis. Hydrogenase activity is calculated from equation (1).

Validation of protocol

This protocol was validated in Kosem et al. (2023). *Applied Catalysis A: General*, DOI: 10.1016/j.apcata.2022.119019.

General notes and troubleshooting

1. To preserve the biological function of O_2-sensitive enzymes, biological samples containing hydrogenase must be protected from air. Therefore, the whole process of cell preparation and experiments must be performed under anaerobic environment or in the glovebox.
2. Kinetic parameters of different hydrogenases from various biological sources can be analyzed by varying the concentrations of methyl viologen as a substrate according to the Michaelis-Menten equation: $v = \frac{V_{max}[S]}{K_m + [S]}$, where v is velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant, and [S] is the concentration of the substrate (methyl viologen).

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Competing interests

The authors declare no competing interests.

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Supplementary information

The following supporting information can be downloaded here:

1. Figure S1. Catalytic sites of different hydrogenases
2. Guideline 1. Safety procedure in handling HCl