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Technical Communication

Formate detection by potassium permanganate for enhanced hydrogen production in *Escherichia coli*

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ABSTRACT

Mutagenesis of *Escherichia coli* for hydrogen production is difficult since there is no high-throughput screen. Here we describe a method for rapid detection of enhanced hydrogen production by engineered strains by detecting formate via potassium permanganate; in *E. coli*, hydrogen is synthesized from formate using the formate hydrogen lyase system.

1. Introduction

To date, many beneficial proteins have been engineered for enhanced catalytic function through DNA shuffling, error-prone PCR, and saturation mutagenesis; for example, epoxide hydrolase [1], amine oxidase [2], alkane hydroxylase [3], toluene-*p*-monooxygenase [4], toluene-*o*-xylene monooxygenase [5], 2,4-DNT dioxygenase [6], and acetyltransferase [7] have all been improved for applications. Success in finding these altered enzymes requires novel high-throughput screening methods that enable the evaluation of many samples (100–1000 colonies/screen), and many of the best methods discriminate based on simple spectrophotometric (color) screening. Unfortunately, protein engineering studies for hydrogenases that would lead to enhanced bacterial hydrogen production have not been developed adequately as there are no high-throughput methods that readily measure hydrogenase activity (either directly or indirectly). One report initiated the evolution of hydrogenase via DNA shuffling [8], but no high-throughput screening method was used and subsequently no improved proteins were discovered.

Escherichia coli produces hydrogen via its formate hydrogen lyase (FHL) system from formate [9]. The FHL system consists of hydrogenase 3 and formate dehydrogenase-H [9], and the FHL enzymes catalyze the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ [10]. Therefore, hydrogen, carbon dioxide, or formate may be suitable targets to measure FHL activity; however, formate should be the best choice compared to other two

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targets since it is not a gas. To date, few methods are available for detecting hydrogen and formate; for example, hydrogen may be detected by chemical reaction with sulfuric acid and potassium permanganate [11] and by colorimetric response by binding of hydrogen to a thin-film WO_3 sensor (chemochromic film) [12], but, these methods require specific materials such as 96-well plates with gaskets for trapping hydrogen gas and chemochromic film. Also, there is a simple method to detect formate by using acetic anhydride [13]; however, this method is difficult for detecting formate with bacterial cells since acetic anhydride also reacts with other salts such as sodium phosphate that is used for suspending cells [14]. Here we report the heuristic technique of a facile method using potassium permanganate to detect formate in biological samples and show that the method is useful by detecting formate with *E. coli* mutants possessing 20-fold higher or lower hydrogen-producing activity.

2. Formate determination by potassium permanganate

Potassium permanganate is a chemiluminescent reagent [15], which is used to detect organic molecules such as catechol, ascorbic acid, and malic acid [16], and has absorption maxima at 525, 545, and 569 nm (Fig. 1). We found that addition of formate (50 and 100 mM) to a 1 mM potassium permanganate solution leads to a significant decrease in absorbance as shown in Fig. 1, and that this inverse relationship between formate concentration and absorbance at 545 nm was linear (Fig. 2); the linear range was from 0 to 105 mM formate (data not shown). The inverse relationship was also observed at 525 and 569 nm. Therefore, potassium permanganate allows one to easily detect formate concentrations in biological solutions by measuring the red color of the unreacted potassium permanganate. The reaction is probably the formation of manganese dioxide by the reduction of potassium permanganate with formate [17]: $2KMnO_4 + 4HCOOH + 2H^+ \rightarrow 2MnO_2 + 4CO_2 + 4H_2O + H_2 + 2K^+$. Note that there is interference by both LB medium and modified complex medium without formate [18] with this novel potassium permanganate assay so phosphate buffer was used to detect formate. Tryptone and yeast extract but not sodium chloride caused interference with the method (data not shown).

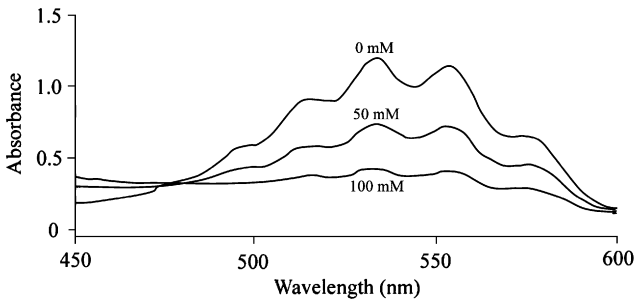

Fig. 1 – Ultraviolet and visible spectra of 0, 50, and 100 mM formate in PMB buffer after addition of 1 mM potassium permanganate within 10 s.

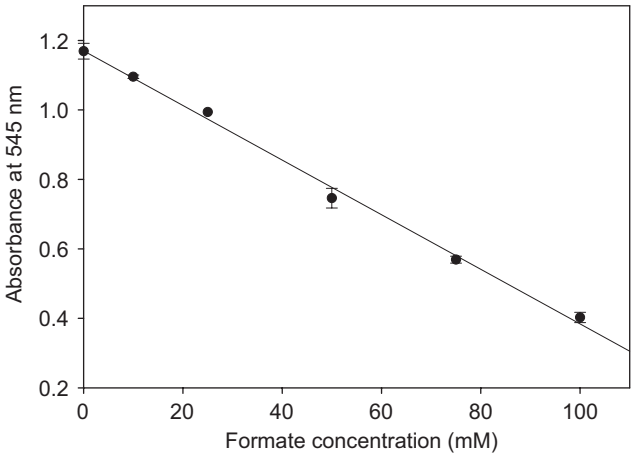

Fig. 2 – Formate calibration curve using the $KMnO_4$ method. Samples (1 mL) containing different concentrations of formate (0, 10, 25, 50, 75, and 100 mM) were mixed with 1 mM potassium permanganate (1 mL), and then the absorbance at 545 nm of the mixtures was measured within 10 s to determine the absorbance. Data are the average ± one standard deviation from two independent measurements.

3. Detection of enhanced hydrogen production

Since *E. coli* cells consume formate as they make hydrogen gas under anaerobic conditions, low formate concentrations are indicative of high hydrogen production rates. To show the relationship between formate and hydrogen production, three strains were utilized, BW25113/pCA24N (wild-type), BW25113 *hyaB hybC hycE*/pCA24N, and BW25113 *hyaB hybC hycA*/pCA24N-FhlA [18]. BW25113 *hyaB hybC hycE*/pCA24N neither produces nor consumes hydrogen since the *hyaB* mutation eliminates hydrogenase 1 activity, the *hybC* mutation eliminates hydrogenase 2 activity [19], and the *hycE* mutation inactivates hydrogenase 3 activity [9]. BW25113 *hyaB hybC hycA*/pCA24N-FhlA is a strain that is also defective in hydrogen uptake (*hyaB hybC*) but overproduces hydrogen due to the *hycA* mutation that inactivates the FHL repressor HycA [20] and due to overexpression of the FHL activator FhlA via plasmid pCA24N-FhlA [18,21]. These strains were grown aerobically at 37 °C overnight in 30 mL of modified complex-formate medium [18] containing 30 μg/mL chloramphenicol to maintain the pCA24N-based plasmids, sparged with nitrogen gas for 5 min, poured anaerobically into 40 mL centrifuge tubes in an anaerobic glove box, and centrifuged (5927 × *g*) for 5 min at 4 °C. The cell pellets were washed once with phosphate mineral buffer (PMB) (pH 6.5, 100 mM) containing 0.039 g/L $Fe(NH_4)2SO_4 \cdot 6H_2O$, 0.172 mg/L Na_2SeO_3, 0.02 mg/L $NiCl_2$, and 0.4 mg/L $(NH_4)_6Mo_7O_{24}$ and resuspended in the same buffer (30 mL). Sealed crimp-top vials (60 mL) were sparged for 5 min with nitrogen, and 18 mL of the cell suspension and 2 mL of 1 M formate were added to the bottles which were incubated at 37 °C anaerobically with shaking for 42 h. The amount of hydrogen generated in the head space was measured as described previously [22]. To measure formate concentrations, 2 mL of

Table 1 – Hydrogen production, final formate concentrations (initial concentration of 100 mM), and formate consumption by *E. coli* cells

Strain	Hydrogen production (μmol/mg protein)		Formate concentration (mM)		Formate consumption (μmol/mg protein)	
	42 h	Relative	42 h	Relative	42 h	Relative
BW25113/pCA24N	5.6 ± 0.6	1	83 ± 4[1] (86.8 ± 0.1)[2]	1 (1)	37 ± 8	1
BW25113 *hyaB hybC hycE*/pCA24N	0.3 ± 0.1	0.05	97 ± 2 (95 ± 2)	1.2 (1.1)	7 ± 5	0.2
BW25113 *hyaB hybC hycA*/pCA24N-FhlA	94 ± 17	16.8	63 ± 8 (71 ± 2)	0.8 (0.8)	154 ± 4	4.2

Total protein concentrations for the *E. coli* strains were 0.22 mg/OD/mL [4]. The amount of hydrogen was determined as described previously [18], and formate concentrations were determined by the $KMnO_4$ method (1) using calibration curves (Fig. 2) made with standard formate and via HPLC (2). HPLC was performed with a Shim-pack SCR-102H column (Shimadzu, Kyoto, Japan) with 5 mM *p*-toluenesulfonic acid as the mobile phase and a flow rate of 0.8 mL/min. Formate was detected with a Shimadzu conductometric detector CDD-6A. Data are the average ± one standard deviation from two independent measurements.

cell suspension was placed into two 1.5 mL tubes and centrifuged at 16000 × *g* for 1 min. The supernatant (1 mL) was mixed with 1 mM potassium permanganate, and the formate concentration was determined by measuring the absorbance at 545 nm within 10 s with a CARY 1E spectrophotometer (Varian, Inc., Walnut Creek, CA). Also, HPLC analysis was performed to corroborate formate detection using $KMnO_4$. The results of HPLC method were identical to those of the $KMnO_4$ method (Table 1); hence, the simple $KMnO_4$ method is useful for detecting formate. In contrast, the pH values of the buffer with the three *E. coli* strains that produced different amounts of hydrogen were not different (data not shown).

As expected [18], BW25113 *hyaB hybC hycA*/pCA24N-FhlA produced 313 times more hydrogen than BW25113 *hyaB hybc hycE*/pCA24N (Table 1), so the use of these three strains allowed us to vary hydrogen synthesis over a wide range. Using the new potassium permanganate assay, formate consumption by these strains was consistent in that BW25113 *hyaB hybC hycA*/pCA24N-FhlA, which produced the most hydrogen, had the lowest formate concentration and the greatest formate consumption rate (Table 1). For the three strains, the formate consumption rates were higher than the hydrogen production rates; this may be due to other formate consumption pathways such as that by formate dehydrogenase-N and formate dehydrogenase-O in *E. coli* [23].

4. Conclusions

Taken together, we demonstrate clearly that formate may be detected by potassium permanganate (Figs. 1 and 2) and may be used with *E. coli* cells to determine hydrogenase activity (Table 1). Hence, this simple method holds promise for protein engineering of hydrogenase to enhance hydrogen production.

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