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High-Throughput Screening Assay for Biological Hydrogen Production

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This paper describes a screening assay, compatible with high-throughput bioprospecting or molecular biology methods, for assessing biological hydrogen (H_2) production. While the assay is adaptable to various physical configurations, we describe its use in a 96-well, microtiter plate format with a lower plate containing H_2-producing cyanobacteria strains and controls and an upper, membrane-bottom plate containing a color indicator and a catalyst. H_2 produced by cells in the lower plate diffuses through the membrane into the upper plate, causing a color change that can be quantified with a microplate reader. The assay is reproducible, semiquantitative, sensitive down to at least 20 nmol of H_2, and largely unaffected by oxygen, carbon dioxide, or volatile fatty acids at levels appropriate to biological systems.

Hydrogen (H_2) is attractive as a renewable energy carrier, particularly when produced by direct biophotolysis of water (biosolar H_2) or by other environmentally sustainable processes.[1,2] Consequently, several laboratories around the world are engaged in research to develop modified strains of bacteria or algae or to find new strains in the environment (bioprospecting), with high H_2 producing capabilities. In either type of research approach, it is likely that thousands of strains must be evaluated to find the few that produce greater quantities of H_2. This is a problematic issue because no high-throughput method for directly detecting H_2 production has heretofore been available.

Established methods for measuring H_2, such as the reverse-polarity Clarke electrode, gas chromatography, or mass spectrometry, require measuring samples one at a time and are time-consuming and labor-intensive.[3–5] A different method has been published in which a chemochromic membrane, impregnated with tungsten oxide and a palladium catalyst, is placed above an agar plate or 96-well microtiter plate containing active microbial colonies, with a filter paper layer separating the two.[6] Evolved H_2 causes bluish/purple spots on the film, but the color changes are transient and diminished by the presence of O_2. Also, H_2 responses are not easily quantified, special equipment is required to prepare the membrane, optimization of the method is difficult, and it does not lend itself well to high-throughput molecular biology methods. The method is reportedly very sensitive, but it has not become widely used. Another, simpler method uses an H_2-oxidizing catalyst and a color indicator together in solution with H_2-producing microbes.[7,8] However, the catalyst can cause microbial inhibition, and non-H_2-mediated color changes can occur, making results from the procedure somewhat unreliable.

The purpose of this research was to develop a sensitive, reliable H_2 screening assay compatible with high-throughput bioprospecting or molecular biology methods. In this paper, we describe use and testing of the assay in a 96-well, microtiter plate format. H_2-producing microbial strains are placed in a lower plate, and a solution containing a color indicator and a catalyst is placed in an upper, membrane-bottom plate. The plates are sealed together, and H_2 produced by cells in each well of the lower plate diffuses through the membrane into the corresponding well in the upper plate, causing a color change that can be quantified spectrophotometrically. Construction of the assay apparatus is simple and all parts, with the exception of the catalyst, are commercially available. The color change is stable and, due to the physical separation of the indicator solution from the cells, is not affected by cellular interactions or nonvolatile metabolites. The assay also is not appreciably affected by oxygen, carbon dioxide, or volatile fatty acids at levels that could be experienced in biological systems.

EXPERIMENTAL SECTION

Arrangement of the Assay Apparatus. An aqueous solution containing the color indicator and catalyst overlays the H_2-producing culture. H_2 evolved by the cells diffuses up through the H_2-permeable barrier into the overlying solution, reacts with the catalyst and color indicator, and causes a visually detectable color change. In a 96-well plate format, we place H_2-producing cultures in the wells of a lower 96-well microtiter plate (1 mL;

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(1) Prince, R. C.; Kheshgi, H. S. *Crit. Rev. Microbiol.* **2005**, *31*, 19–31.
(2) Ghirardi, M. L.; Posewitz, M. C.; Maness, P. C.; Dubini, A.; Yu, J. P.; Seibert, M. *Annu. Rev. Plant Biol.* **2007**, *58*, 71–91.
(3) Appel, J.; Phunpruch, S.; Steinmuller, K.; Schulz, R. *Arch. Microbiol.* **2000**, *173*, 333–338.
(4) Cournac, L.; Mus, F.; Bernard, L.; Guedeney, G.; Vignais, P.; Peltier, G. *Int. J. Hydrogen Energy* **2002**, *27*, 1229–1237.
(5) Hansel, A.; Lindblad, P. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 153–160.
(6) Ghirardi, M. L.; Zhang, J. P.; Lee, J. W.; Flynn, T.; Seibert, M.; Greenbaum, E.; Melis, A. *Trends Biotechnol.* **2000**, *18*, 506–511.
(7) Katsuda, T.; Ooshima, H.; Azuma, M.; Kato, J. *Chem. Eng. Symp. Series, 57: Horizon Biochem. Eng.* **1997**, 81–85.
(8) Katsuda, T.; Ooshima, H.; Azuma, M.; Kato, J. *J. Biosci. Bioeng.* **2006**, *102*, 220–226.

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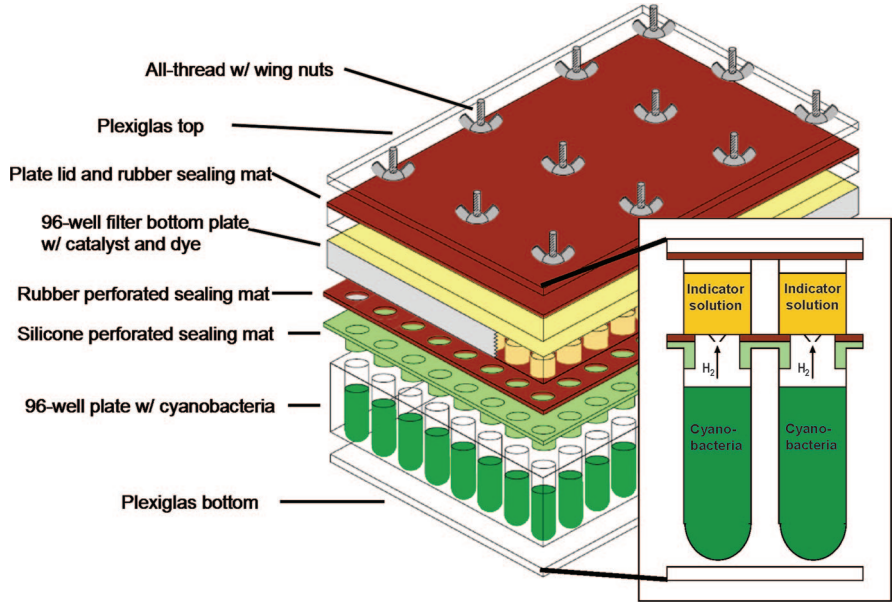

Figure 1. Diagram of the high-throughput screening assay apparatus.

Nunc, Rochester, NY) with the indicator solution above it in a 96-well, membrane-bottom plate (0.2 μm PTFE filter plates, Pall, Ann Arbor, MI) (Figure 1). We seal the two plates together and prevent well-to-well crosstalk with a molded silicone plate sealer (BioTech Solutions, Vineland, NJ) and a butyl rubber gasket, both perforated above each well. The top of the upper plate is sealed with an adhesive microtiter plate sealing mat (USA Scientific Titer Tops, Ocala, FL), butyl rubber gasket, and microtiter plate lid. The plates are layered between two, $^1/_8$ in. thick pieces of Plexiglas and clamped together using 12 pieces of all-thread with locknuts on the bottom and wing nuts on the top (Figure 1). Holes are predrilled through all layers of the apparatus, in between the wells, so no wells are sacrificed.

Incubation and Quantification. We incubate the apparatus in conditions conducive to H_2 production by the microbial cultures. For the experiments described below, we incubated cultures of *Synechocystis* sp. PCC 6803 *ndhB*-deficient mutant strain M55 for 5.5 days at 30 °C with cycles of 8 min of light (from below; cool-white fluorescent bulbs; 200 μE m^{-2} s^{-1} of PAR), followed by 52 min of darkness. This light/dark cycle was chosen to maximize the short-lived hydrogen production (<10 min for the M55 mutant of *Synechocystis* sp. PCC 6803 upon illumination after dark, anoxic incubation) over a 24 h period. After incubation, we removed the tops, transferred 200 μL from each well of the upper plate to an optically clear 96-well plate (BD Falcon, San Jose, CA), and read absorbance of each well at 450 nm in a spectrophotometric microplate reader (Biorad Ultramark EX, Hercules, CA). We used this approach to evaluate several color indicators, catalysts, and H_2-permeable membranes (Table 1). So far, the combination we have found to be most sensitive and reliable (data not shown) is a water-soluble tetrazoleum color indicator (WST-3, Dojindo, Gaithersburg, MD), sulfonated Wilkinson's catalyst (99.99% pure, Acros, Morris Plains, NJ), and PTFE membrane material.

Sulfonation of Wilkinson's Catalyst. Water-soluble, sulfonated Wilkinson's catalyst was made by the method of Katsuda et al. (1997), modified as follows.[7] One gram of Wilkinson's catalyst (tris(triphenylphosphine) rhodium chloride) was sealed in a 10 mL Erlenmeyer flask with 4 mL of fuming sulfuric acid and gently shaken overnight at 45 °C. The solution was neutralized with 10 N NaOH and passed through a filtering syringe with a 1.5 μm high retention borosilicate glass microfiber filter (Whatman, Kent, U.K.) to remove the red-tinged, white, sodium sulfate precipitate formed as a neutralization byproduct. The filtrate, containing sulfonated Wilkinson's catalyst, was frozen in a dry ice and isopropanol bath, then lyophilized. The freeze-dried powder was resuspended in methanol to dissolve the sulfonated Wilkinson's catalyst, the solution was centrifuged to pellet residual sodium sulfate, and the red methanol solution, containing the sulfonated Wilkinson's catalyst, was collected. The sodium sulfate pellet was re-extracted with methanol until the red color was gone, leaving a white pellet. The methanol was evaporated off in a fume hood leaving behind the red solid that is the sulfonated Wilkinson's catalyst (>90% yields routinely achieved). The solid Wilkinson's catalyst was then reconstituted in DI water to a concentration of 10 mM and stored under a nitrogen atmosphere at 4 °C in the dark.

Table 1. Color Indicators, Catalysts, and Membranes Tested (in no Particular Order)

color indicators	WST-1, 3, 5, 9, 11; XTT, methyl orange, methylene blue; methyl purple
catalysts	sulfonated Wilkinson's catalyst; sulfonated iridium cyclooctadiene triphenylphosphine; sulfonated rhodium cyclooctadiene triphenylphosphine; sulfonated ruthenium triphenylphosphine; *Azotobacter vinlandii*; *Ralstonia eutropha*
membranes	PTFE; nylon; nitrocellulose; parafilm; plastic membrane

Safety Considerations. Normal care should be taken when handling strong acids and bases. The addition of fuming sulfuric acid to Wilkinson's catalyst and the subsequent neutralization with sodium hydroxide should both be performed in a chemical fume hood.

Preparation of Assays. WST-3 and water soluble Wilkinson's catalyst were diluted and mixed with DI water at selected

concentrations (see Assay Optimization), and 300 μL were placed in each of the 96 wells of a membrane-bottom plate. Cells were concentrated to 1×10^9 cells per mL by centrifugation and resuspended in BG-11 media containing 35 mM HEPES buffer and 80 mM sodium bicarbonate at pH 10. A volume of 1 mL of cells, or 1 mL of media as negative control, was placed in the appropriate wells of the lower 96-well plate. Both plates were placed in a hypoxic glovebag (0.1% O_2, Coy Laboratory Products, Grass Lake, MI) for 2 h before being assembled, as described above. All tests were performed in triplicate.

Optimization of Color Indicator Solution. We conducted preliminary tests to identify optimum color indicator and catalyst concentration ranges. A response surface methodology (RSM) approach was used for experimental design and data analysis, with a rotatable (axial distance, $\alpha = 1.682$), inscribed central composite design (five levels of each variable) with two center points and three replicates of each condition. We used the software program JMP Statistical Discovery 6.0.0 from SAS (Cary, NC) to fit the standard second-order equation:

$$\hat{y} = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i<j} \sum \beta_{ij} x_i x_j \qquad (1)$$

where k is the number of variables ($k = 2$, catalyst and color indicator concentrations, in this study), and β_0, β_j, β_{jj}, and β_{ij} are the intercept, linear, quadratic, and interaction coefficients, respectively.

The matrix form of the equation is useful for analyzing the shape of the response surface:

$$\hat{y} = b_0 + \mathbf{x'b} + \mathbf{x'Bx} \qquad (2)$$

where b_0, $\mathbf{b}$, and $\mathbf{B}$ are estimates of the intercept, linear, and second-order coefficients, respectively, and

$$\mathbf{x} = \begin{bmatrix} x_1 \\ x_2 \\ \vdots \\ x_k \end{bmatrix} \quad \mathbf{b} = \begin{bmatrix} b_1 \\ b_2 \\ \vdots \\ b_k \end{bmatrix} \quad \mathbf{B} = \begin{bmatrix} b_{11} & b_{12}/2 & \cdots & b_{1k}/2 \\ & b_{22} & \cdots & b_{2k}/2 \\ & & \ddots & \vdots \\ sym. & & & b_{kk} \end{bmatrix}$$

To solve for the stationary point, the equation was differentiated and set to zero.

Sensitivity to H_2. We determined catalyst and color indicator sensitivity to H_2 in 2 mL screw cap glass GC vials with PTFE-lined septa (VWR, West Chester, PA). WST-3 and catalyst were diluted to selected concentrations with DI water, 300 μL were placed in each GC vial, and the mixtures were incubated in the hypoxic glovebag (0.1% O_2) for 2 h. The vials were then capped, removed from the glovebag, and each was injected with an appropriate quantity of ultrahigh-purity H_2, inverted, and incubated in the dark at 30 °C. Following incubation for the selected time period, the vials were opened and 200 μL were placed in a 96-well plate and absorbance readings were made at 450 nm with the microplate reader. All vial tests were performed in triplicate.

Effects of Biologically Relevant Gases. Tests to determine possible affects of biologically relevant gases, such as O_2 and CO_2, on assay response were performed in glass GC vials, as described above. Once capped and removed from the glovebag, the vials were injected with the appropriate quantities of ultrahigh-purity gas(es), then incubated and analyzed as described above. To determine the quantity of O_2 and CO_2 to add, we estimated the amount that would be produced by *Synechocystis* sp. PCC 6803 over 5 days and doubled it. All vial tests were performed in triplicate.

Table 2. RSM Experimental Design Showing the Coding of the Factors Color Indicator Concentration (First Factor) and Catalyst Concentration (Second Factor)[a]

pattern	color indicator concentration (mM)	catalyst concentration (mM)	response
a0	0.1	2.55	0.068
a0	0.1	2.55	0.076
a0	0.1	2.55	0.038
−−	0.818	0.818	0.162
−−	0.818	0.818	0.124
−−	0.818	0.818	0.158
−+	0.818	4.282	0.075
−+	0.818	4.282	0.146
−+	0.818	4.282	0.1
0a	2.55	0.1	0.207
0a	2.55	0.1	0.169
0a	2.55	0.1	0.193
00	2.55	2.55	0.196
00	2.55	2.55	0.23
00	2.55	2.55	0.236
00	2.55	2.55	0.227
00	2.55	2.55	0.194
00	2.55	2.55	0.238
0A	2.55	5	0.16
0A	2.55	5	0.269
0A	2.55	5	0.178
+−	4.282	0.818	0.277
+−	4.282	0.818	0.261
+−	4.282	0.818	0.286
++	4.282	4.282	0.178
++	4.282	4.282	0.248
++	4.282	4.282	0.207
A0	5	2.55	0.233
A0	5	2.55	0.206
A0	5	2.55	0.274

[a] In the pattern layout, with "0" for midrange concentration, "−" and "+" for low and high concentration, respectively, and "a" and "A" for low and high axial concentration, respectively. The actual concentration tested and the corresponding response is also shown.

Culture Growth Conditions. Because it produces more H_2 than the wild-type strain, we used cultures of *Synechocystis* sp. PCC 6803 strain M55 in these experiments.[9] Cultures were grown at 30 °C in BG-11 medium, supplemented with 20 mM sodium bicarbonate, under constant illumination (50 μE m^{-2} s^{-1}), and harvested in mid log-growth phase.

RESULTS AND DISCUSSION

Assay Optimization. Table 2 shows the coded experimental design, the concentrations tested at each point, and the response, defined as the change in absorbance between the cyanobacterial sample and its respective media control, with each point being run in triplicate. The concentration range for both the catalyst and the color indicator was 0.1−5.0 mM. The R^2 of the response surface regression was 0.803. Response of the color indicator solution to H_2 (illustrated in Figure 2) was found to be maximal with critical values of 4.3 mM for color indicator and 0.94 mM for

(9) Cournac, L.; Guedeney, G.; Peltier, G.; Vignais, P. M. *J. Bacteriol.* **2004**, *186*, 1737–1746.

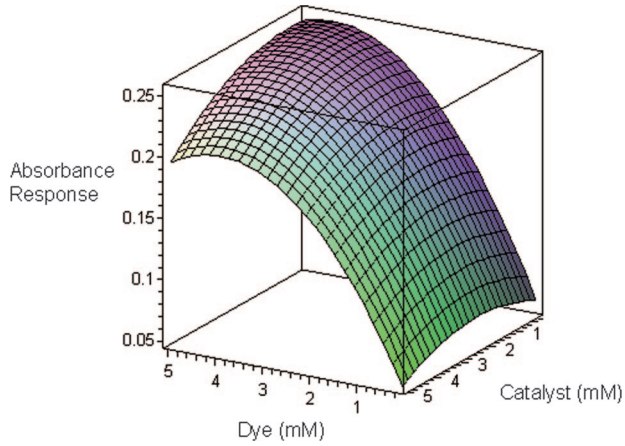

Figure 2. 3-D figure showing response on the vertical axis vs dye and catalyst concentrations.

catalyst. These optimum values were used in the subsequent vial tests to semiquantitatively relate absorbance change to amounts of H_2, to determine the sensitivity of the assay to H_2, and to examine potential assay inhibition by other biologically relevant gases, such as O_2 and CO_2.

Hydrogenation of WST-3. Figure 3 shows the postulated reaction involved in the hydrogenation (reduction) of the color indicator WST-3 by H_2 gas mediated by the water soluble form of Wilkinson's catalyst. To demonstrate that the change in color taking place was due to this reaction, we set up duplicate GC vials containing the appropriate concentration of color indicator and catalyst by themselves and together under a nitrogen atmosphere. One set received no treatment while the other set was injected with 4.1 μmol of H_2 gas. After 1 day of incubating at 30 °C, the absorbance of each vial was scanned from 300−500 nm with a spectrophotometer (Thermo Scientific GENESYS 10uv, Waltham, MA). In the vials containing both catalyst and color indicator, Figure 4 shows a significant absorbance increase when H_2 was present. No absorbance increase was observed when H_2 was injected into vials containing only color indicator or catalyst (data not shown). Figure 4 also indicates substantial absorbance increases with the Wilkinson's catalyst alone at wavelengths less than about 430 nm and similarly with WST-3 alone at wavelengths less than 410 nm. This suggests a range from roughly 450−500 nm is best suited for measuring absorbance changes in the catalyst and color indicator solution due to catalyst-mediated color indicator reduction from H_2 gas. With the Ultramark automated plate reader, we found that a 450 nm filter was optimal (data not shown). Because the catalyst contributes to the background absorbance levels, it is important to optimize the concentrations of both catalyst and its respective color indicator in order to achieve both a high response and sensitivity of the assay to H_2.

Sensitivity of Assay Response to H_2. To evaluate the response of the assay to H_2, we produced a standard curve with triplicate H_2 concentrations over a range of 0−0.82 μmol. Linear regression yielded an R^2 of 0.968, with a p-value of 0.0027 and a regression equation of $y = 0.363x + 0.468$, where x is the amount of H_2 (μmol) and y is the change in absorbance.

Similarly, using 0−41.2 nmol of H_2 another set of tests was performed to evaluate the H_2 detection limit of the assay. The lowest concentration tested that yielded a response at least 1 standard deviation above the control was 20.6 nmol. The upper limit of hydrogen concentration in the assay is constrained by a theoretical limit of 2.15 mM, based on the optimized concentration of dye and the reaction shown in Figure 3. To use the screening assay with a species that would produce more than 2.15 mM H_2, this upper limit could be changed by adjusting the dye and catalyst concentrations.

In other tests, absorbance changes of the catalyst and color indicator after injection of various concentrations of H_2 were analyzed over time in GC vials (data not shown). There were no statistically significant differences between the earliest measurements at 0.5 days and all subsequent measurements, indicating that the catalyzed reaction in the color indicator was complete in half a day or less, and that the H_2/catalyst/color indicator reaction kinetics are appreciably faster than the rate of H_2 evolution from *Synechocystis* sp. PCC 6803.

Effects of Biologically Relevant Gases and Organic Acids on Assay Response. Apart from H_2, the two major gases produced and consumed by H_2-producing cyanobacteria and algae are O_2 and CO_2. Because catalysts using transition metals may be affected adversely by one or both of these gases, it was important to determine possible effects of the gases on WST-3 and the sulfonated Wilkinson's catalyst.[10,11] The tested ranges of O_2 and CO_2 quantities were chosen to bound the amounts of the gases expected to be produced by *Synechocystis* PCC 6803 under our assay conditions.

In the absence of H_2, Figure 5 shows that O_2 had little or no effect on absorbance readings of the color indicator solution. With 2.06 μmol of H_2 present, increasing O_2 amounts decreased assay response somewhat, from a mean absorbance of 0.91 without O_2 to 0.76 (16% decrease) with 4.1 μmol of O_2. Because an important goal is to discover or create microbial species or strains with a hydrogenase capable of operating at atmospheric O_2 concentrations, we performed similar vial tests to determine assay response under such conditions. A 15% decrease in response was observed when the vials were degassed as normal and then treated by replacing the headspace gas with 21% O_2, whereas a 45% decrease in response was observed when the vials were exposed to atmospheric conditions and not degassed at all prior to capping and the addition of H_2 (data not shown). These results imply that the assay is capable of performing under atmospheric-like oxygen levels but that optimal results are obtained when the indicator solution is first degassed with N_2.

Unlike O_2, CO_2 caused slightly decreased response of the color indicator solution even when no H_2 was present, from 0.51 without CO_2 to 0.40 (22% decrease) with 4.1 μmol of CO_2. Because this effect occurred in the absence of H_2, it cannot be attributed to inhibition of an H_2-associated reaction. With 2.06 μmol of H_2 present, increasing amounts of CO_2 decreased assay response by up to about 16%, essentially identical to the effects observed with O_2. The cause(s) of this decreased response is/are not clear. Because we use the assay only semiquantitatively as a screening tool, these slight decreases in assay response in the presence of O_2 or CO_2 have not been a major concern in our research. Also,

(10) Osborn, J. A.; Jardine, F. H.; Young, F. H.; Wilkinson, G. *J. Chem. Soc. A.* **1966**, 171.

(11) Osborn, J. A.; Wilkinson, G. *Inorganic Syntheses, Reagents for Transition Metal Complex and Organometallic Syntheses*, Vol. 28; Angelici, R. J., Ed.; Wiley: New York, 1990; pp 77−79.

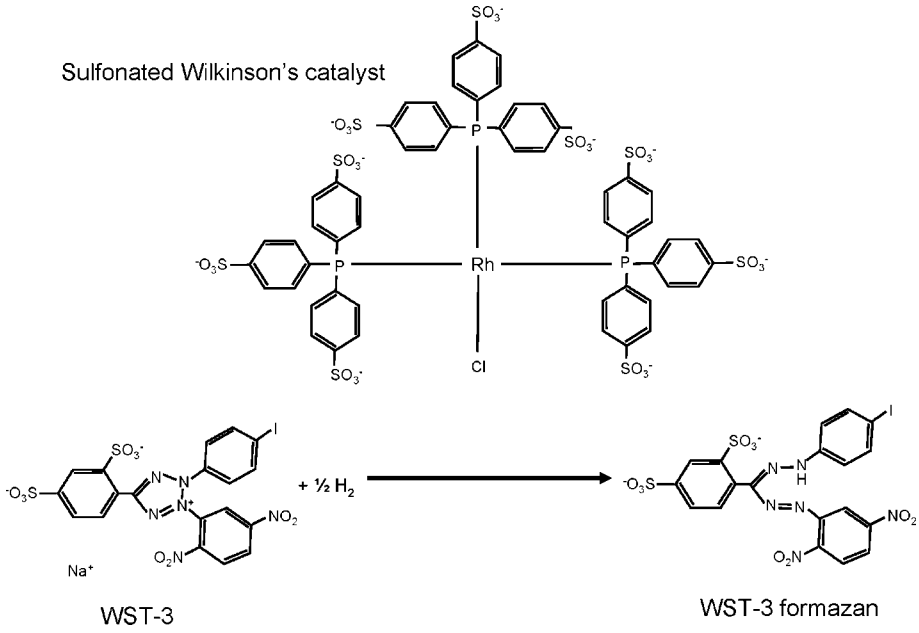

Figure 3. Reaction of WST-3 with H_2 to form WST-3 formazan.

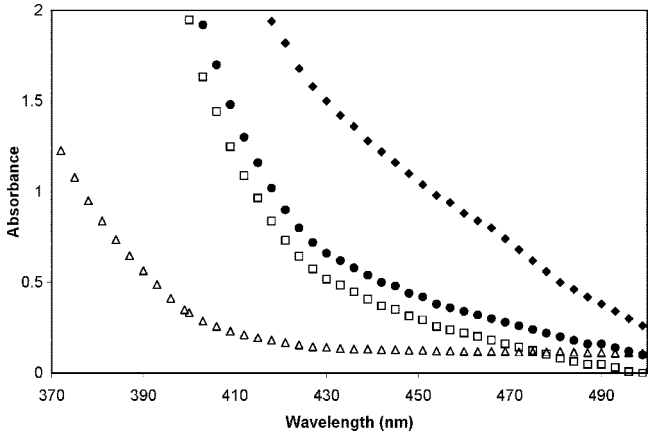

Figure 4. Absorbance scan of WST-3, sulfonated Wilkinson's catalyst, and WST-3 and sulfonated Wilkinson's catalyst together with and without H_2. WST-3 dye only (△), soluble Wilkinson's catalyst only (□), WST-3 and soluble Wilkinson's catalyst without hydrogen (●), and WST-3 and soluble Wilkinson's catalyst with 4.1 μmol of hydrogen (◆).

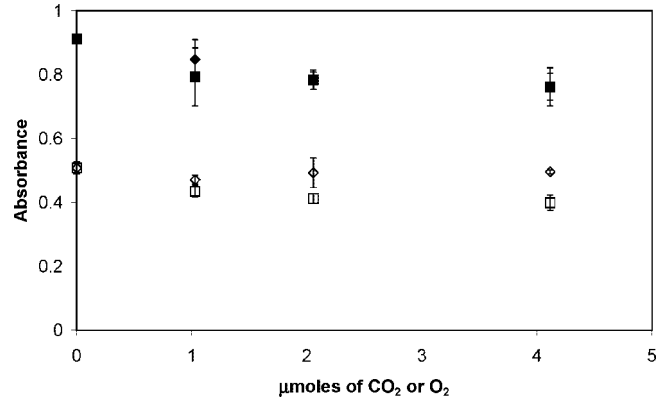

Figure 5. Effect of biological gases on color indicator and catalyst in GC vial tests. All tests performed in triplicate; error bars indicate 1 standard deviation. Treatments were WST-3 and soluble Wilkinson's catalyst with the following: 0−4.1 μmol of CO_2, 0−4.1 μmol of O_2. Legend: □, CO_2 only; ◇, O_2 only; ■, $CO_2 + H_2$; ◆, $O_2 + H_2$.

the extent to which the experimental procedure could have played a role in diminished responses is also unclear; under the test conditions, vials received a single injection of gas(es) at the beginning of the experiment whereas under the conditions of a biological assay, the gases would be much more slowly produced and/or consumed. In summary, because the presence of relatively high concentrations of CO_2 or O_2 can decrease assay response, it is necessary for users of the assay to consider the production and/or consumption of these gases during an experiment.

Though *Synechocystis* sp. PCC 6803 does not produce substantial amounts of fermentation products including volatile fatty acids (VFAs), the same is not true of some other H_2-evolving species. Therefore, to evaluate potential effects on the screening assay caused by major products of fermentation, we tested the following compounds at 1 μM−100 mM in GC vial tests, both with and without H_2: acetate, butyrate, formate, propionate, and ethanol. These experiments showed ethanol to have no effect at any concentration tested and the organic acids to have no effect at concentrations below 100 μM (data not shown). Possible effects of methane on performance of the screening assay were also tested at concentrations between 10 μM and 10 mM. No statistically significant differences from controls (1 standard deviation) were observed at any methane concentration (data not shown). Because the physical layout of the apparatus separates the color indicator solution from the active biological component, fermentation products that volatilize into the headspace of the lower plate would have to diffuse through the membrane into the color indicator solution in quantities exceeding 100 μM in order to be of concern to the reliability of the assay. Researchers contemplating use of the screening assay should consider whether or not such levels of volatile compounds or gaseous metabolites could be produced in their experiments.

Attempts to Compare the Screening Assay with Established Methods for Measuring Hydrogen. We have attempted to compare the screening assay quantitatively with three other methods for measuring hydrogen: injecting headspace gas from

hydrogen-producing cultures (both batch and chemostat) into a gas chromatograph with a thermal conductivity detector; monitoring aqueous-phase hydrogen concentration with a reverse-polarized Clarke-type electrode; and analyzing production and consumption of hydrogen and other dissolved gases over short time spans under a variety of conditions with a membrane inlet mass spectrometer (MIMS). So far we have found it impossible to carry out straight side-by-side comparisons of the screening assay with these other methods because it functions so differently.

Evolved hydrogen that diffuses through the membrane into the upper plate is captured by reacting with the color indicator, rendering it unavailable to the reversible hydrogenase enzyme. Thus what is measured is cumulative hydrogen evolution into the color indicator, usually over a period of 3−5 days. In contrast, in assays conducted for a similar time period in the presence of the bacteria, regardless of the method of hydrogen measurement, hydrogen is both produced and consumed by the *Synechocystis* sp. PCC 6803 reversible hydrogenase. We have attempted to conduct the comparisons both with and without methyl viologen and dithionite, a traditional method for assessing the activity of hydrogenase enzymes, where an artificial condition is created by sodium dithionite removal of dissolved oxygen and chemical reduction of methyl viologen and provision of electrons to the hydrogenase via a not-well-characterized process. None of the attempts have been successful, some because of the enzyme bidirectionality issue mentioned above and some because of the artificial nature of the methyl viologen/dithionite approach, which we cannot recreate in our assay plates. We also have attempted to create an "internal standard" by placing aluminum and sodium hydroxide in some of the wells in the bottom plate of the assay apparatus. So far these efforts have been thwarted by the speed of the hydrogen-evolving reaction that takes place between the aluminum and the sodium hydroxide; much of the produced hydrogen escapes during the few minutes that is required to seal up the assay apparatus.

So far, the best results have been obtained by injecting known amounts of hydrogen gas into GC vials containing the catalyst and dye used in our assay. This approach has allowed us to quantify the response of the color indicator to hydrogen and is the method by which we ascertained the detection limit and sensitivity of the color indicator. Also, on the basis of comparisons of MIMS results in our laboratory with published results by Cournac et al. (2004), we have found that the observed assay response is in line with expected values obtained from the same organism.[9] Finally, in both GC vial assays and bioreactor experiments using mutant strains of hydrogen-producing bacteria, we have observed that strains shown to produce more hydrogen by these methods also are seen to produce more hydrogen in the screening assay.

SUMMARY AND CONCLUSIONS

A semiquantitative, high-throughput assay has been developed that uses color change (absorbance increase) of an indicator solution to show H_2 production. The color indicator solution contains a catalyst (sulfonated Wilkinson's) and a color indicator (WST-3) and is separated from the biological sample by a gas space and a PTFE membrane. With optimization of the color indicator, the assay is sensitive to low levels of H_2. Biologically relevant gases, specifically O_2 and CO_2, can cause slight decreases in the assay response to H_2, but volatile fermentation products, namely, ethanol, formate, acetate, propionate, butyrate, and methane, at levels up to at least 100 μM cause no substantial effects on assay response.

The assay was developed to provide a reliable, versatile method for screening H_2 production by large numbers of microbial or algal cultures. It can be adapted to various physical configurations and diverse applications, for example, screening of mutant strains (e.g., from directed evolution experiments) or environmental samples/isolates (e.g., from bioprospecting studies) for H_2 production. The assay can also be used for rapid evaluation of the effects of various factors, such as pH, P, S, C, and N, on production of H_2.

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