Establishment of Control Parameters for in Situ, Automated Screening of Sustained Hydrogen Photoproduction by Individual Algal Colonies

D. A. Graves, M. E. Reeves, and E. Greenbaum
Chemical Technology Division, Oak Ridge National Laboratory; Oak Ridge, Tennessee 37831

ABSTRACT

An apparatus was constructed which allowed automated screening of individual microalgal colonies for sustained ability to photoevolve H2 during anaerobic photosynthesis. The main components of this apparatus were a microcomputer, a He-Ne laser mounted on a computer-controlled X-Y translation stage, a flow-through chamber which contained an agar plate of colonies, and a H2 detector which interfaced with the microcomputer for data collection. The system was capable of detecting a minimum production rate of 1 nanomole of H2 per hour per colony and provided an efficient means of screening relatively large numbers of algal colonies. Examination of the effect of the spacing of colonies on the agar plate, light intensity, stability of colonies within a screening period, colony age, chlorophyll content, and colony size on H2 yield indicated that, under optimum conditions, yields from genetically uniform colonies varied by no more than a factor of 2 in their H2-producing ability. Therefore, colonies of algae whose H2 yields lie outside this intrinsic twofold variability can be identified and selected as natural variants or mutants. A description of the construction and of the apparatus is presented, and the experimental results used to establish the control parameters for Chlamydomonas reinhardtii colonies are discussed.

Hydrogen evolution by microalgae represents a unique variant of photosynthesis in which the energy-rich photoprodut is molecular H2, rather than reduced CO2. Since the pioneering discovery of Gaffron and Rubin (8), several genera of microalgae have been shown to be capable of photoevolving H2 under anaerobic conditions (3, 4, 23). The recent awareness of somaclonal variation in clones of higher plants (7) and the finding that repeated cycles of anaerobic photosynthesis and regrowth of Chlamydomonas reinhardtii improved both H2 yield and tolerance to anaerobiosis (18, 22) prompted interest in inherent variability in H2 photoevolution among individual colonies of microalgae.

Prior techniques for analyzing individual members of an algal population required growth of each clone in liquid and analysis of the clones individually. Although amperometric electrode and manometric techniques for measuring H2 have previously been reported (2, 4, 6, 12, 17, 20, 21), they are incapable of measuring sustained steady state rates of H2 production. Since a period of 3 h is required for adaptation of light-driven H2 evolution and 1 to 2 h is required for H2 photoevolution to approach steady state (plus additional time for reestablishment of baseline conditions) in the case of liquid culture, only one clone can be screened per measuring apparatus per day. To circumvent this lengthy and tedious process, an apparatus was constructed and interfaced to a microcomputer which allowed single colonies on an agar plate containing multiple colonies to be screened sequentially for their H2-producing ability. This apparatus, particularly the H2-sensing component (10, 13), is not hindered by the inherent limitations of other H2 detection methods. It can be used to examine relatively large numbers of algal colonies for inherent variation in their steady state H2-producing ability, to evaluate putative mutants that are defective or enhanced in H2-producing ability, and to compare H2 photoevolution from cells growing on solid versus liquid medium.

MATERIALS AND METHODS

Chlamydomonas reinhardtii (University of Texas Culture Collection, strain 90 [int-]) was grown on minimal medium, pH 6.8 (18), under photoautotrophic conditions at about 12 W m-2 and 25°C with 14 h/10 h light-dark cycles. Liquid cultures were diluted and plated on minimal agar plates to give 10 to 30 colonies per plate. The Chl content of single colonies was determined by excising a colony from the plate along with a small piece of agar and placing it into a 1.5-ml microcentrifuge tube with 0.5 ml of methanol. The colony was usually white after 1 h of dark incubation at room temperature in methanol. Chl extraction from post-screened colonies was more difficult but was achieved by heating the extraction mixture to 65°C for 5 min. Chl was quantitated using the extinction coefficients of MacKinney (15, 18). Colony diameter was determined by using an ocular micrometer and a dissecting microscope. The algae were routinely checked for contamination by plating dilutions of liquid cultures onto tryptic soy agar.

One 100 × 15 mm Petri dish was housed in a two-piece glass chamber. The top of the chamber was recessed to enclose the dish and grooved around the perimeter to accept an O-ring. Inlet and outlet ports for gas exchange were built into the top. The bottom was a flat piece of glass. An agar plate was inverted and placed on the bottom, and the top was positioned over the plate and sealed with an O-ring. The entire assembly was sealed and held in place with three bolts that passed through the top of the support frame, the glass plate, and a clamp on the top (Fig. 1, top, bottom). The chamber was connected to a regulated H2 supply and vented through the H2 sensor. The He was humidified by bubbling it through a cell containing doubly distilled H2O.
FIG. 1. Assembled sample chamber containing an inverted plate of algal colonies. Top, An overhead view of the assembled chamber showing the clamp used to secure the chamber and the position of the plate of colonies. The small points of light near the laser beam are photographic artifacts. Bottom, A lateral view showing the support frame, the sample chamber, and the front surface mirror connected to the laser for reflecting the beam to a colony.
Before being passed through the chamber, He flow was held constant at \(25 \text{ ml min}^{-1}\).

Colonies were individually irradiated using a He-Ne laser (model 80-2HA, Coherent, Palo Alto, CA [633-nm wavelength]). The laser beam was positioned over a colony using a X-Y translation stage (Micro Controle) adapted to carry the laser. As the \(H_2\) analyzer was sensitive to moisture and pressure changes, moisture traps consisting of \(1 \times 3\) molecular sieves (Linde Corp.) immersed in ice were installed upstream from the analyzer. The copper tubing connecting the various pieces of \(13 \times 3\) molecular sieves (Linde Corp.) immersed in ice were installed upstream from the analyzer. The copper tubing was made using Swagelok connectors, glass and brass O-ring fittings, copper tubing, Viton O-rings, and spring clamps with locking nuts. The gas sensor was a Snoopy Ethylene Detector (Bio-gas Detector Corp., Okemos, MI) modified for continuous gas flow measurements. This technology, employing a Figaro 812 gas sensing SnO2 semiconductor, has been previously applied for the detection of combustible gases in continuous flow systems (10, 13, 18) and gas chromatographic configurations (13, 16). The gas sensor/calibration cell assembly used for measuring the photoproduced \(H_2\) was similar to that described earlier (9, 10).

Data collection and laser positioning were automatically performed using a Hewlett-Packard 85 microcomputer via an IEEE-488 interface. A Klinger programmable stepper motor controller was driven by the computer to position the laser on and off selected colonies at predetermined time intervals. The X-Y coordinates for each colony to be screened were entered into the computer by manually positioning the laser beam on the colony; the coordinates were transmitted from the stepper motor controller to the computer over the IEEE-488 interface. The analog output from the \(H_2\) sensor was displayed on a Keithley model 197 multimeter and collected over the IEEE-488 bus. Data were recorded and stored on tape cassettes and also recorded directly from the multimeter's analog output on a chart recorder. (See Figure 2 for a schematic representation of the apparatus.)

After a plate of colonies had been placed in the chamber and the system sealed, the colonies were subjected to 4 h of darkness at room temperature for adaptation. During this time, calibration data were collected by supplying current steps to the electrolysis cell and recording the sensor response for each step. Calibrations were typically run with 9, 7, 5, and 3 \(\mu\)amp for 45 min at each current. Following dark adaptation, single colonies were screened with the laser beam positioned on the colony for a minimum of 1 h. After the light-on period, the laser was moved off the colony to a small piece of black felt for light off. Baseline conditions were reestablished during the light-off time (minimum dark time, 1 h). The \(H_2\) yield of single colonies was quantitated using the calibration data generated by electrolyzing water. Software was written to plot the raw data, calculate the rates of \(H_2\) evolution, and determine the integrated yield of \(H_2\) during each light-on period. This software, written in BASIC language, is available upon request.

RESULTS

\(H_2\) photoevolution from individual algal colonies was easily measured using the described apparatus. Photoevolution rates of less than 1 nmol \(H_2\ h^{-1}\) were measurable with the gas analyzer. Figure 3 shows typical calibration data and the \(H_2\) production rate by three individual colonies. The integrated experimental yield of \(H_2\) during calibration is in close agreement with the theoretical yield of 336 nmol calculated using 0.00518 nmol \(H_2\ s^{-1} \mu\)amp\(^{-1}\). Integrated yields from the calibration data were within 5% of the theoretical yield, indicating the accuracy of this analysis. Another significant feature of the measurement system reflected in the data shown in Figure 3 is the low noise level. The shape of the \(H_2\) evolution profiles accentuates the difference between these measurements and those made with amperometric electrode techniques. The ability to examine \(H_2\) evolution for extended time periods gives sustained photohydrogen evolution rates rather than transient rates determined by short-term experiments using amperometric electrodes.

Several factors were examined which could affect real or apparent \(H_2\) photoevolution from colonies. The following factors were considered in analyzing and interpreting the data: the age, Chl content, and size of colonies; light intensity; stability of the colonies under anaerobiosis; the effect of scattered light on adjacent colonies; and the survivability and cloning of screened algal colonies.

Scattered Light. The effect of scattered light on stimulating \(H_2\) evolution from adjacent colonies was examined to determine the minimum separation between colonies so that neighboring colonies...
colonies would not contribute significantly to the H₂ yield of the irradiated colony. A plate containing a single colony was placed in the apparatus, and the translation stage controller was programmed to increment the laser beam toward the colony. Each incremental step was held in position for 1 h followed by 1 h of darkness. The last position was directly on the colony to determine maximum yield. Table I shows the results of these experiments using the unattenuated laser beam and the beam attenuated with a No. 1 neutral density filter to 10% of the maximum power. The maximum yield was taken as 100% and the yields detected at various distances from the colony are indicated as a percentage of the maximum. The data indicate that the colonies should be separated by >1 cm when using the unattenuated laser beam in order to avoid stimulating neighboring colonies from producing H₂. Additionally, this experiment was a measure of light scattering by agar, which was more severe than scattering by colonies due to absorption of much of the incident light.

Stability of Colonies. The stability of H₂ evolution from a single colony was investigated so that colonies would not be screened after their H₂ producing ability had begun to decline significantly. A single 14-d-old colony was studied in response to 1-h light-on and 1-h light-off cycles for over 100 h (Fig. 4). Both the dark time prior to the first irradiation and the dark time between successive irradiations of the same colony influenced the response of a colony. Figure 5 suggests that the first irradiation should occur within the first 20 to 24 h of dark anaerobiosis. This conclusion was consistent with the data in Figure 4. Figure 5 also indicates that the dark time prior to the first irradiation has a minimal effect on H₂ photoevolution if it occurs within the first 20 to 24 h of dark anaerobiosis. The decline in yields seen after 20 to 24 h appears to be a reflection of the biological

Table 1. Effect of Scattered Light on Hydrogen Photoproduction by Adjacent Colonies

<table>
<thead>
<tr>
<th>Power (mW)</th>
<th>Maximum H₂ (nmol/h)</th>
<th>Maximum H₂/Distance from Colony (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1.5</td>
<td>13.3</td>
<td>100</td>
</tr>
<tr>
<td>0.15</td>
<td>4.7</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 3. Typical calibration data and data from three individual colonies. Calibration (time 0–4 h) was performed with 9, 7, 5, and 3 μAmp, respectively, for 45 min at each current. The yield of hydrogen during calibration was 336 nmol based on 0.00518 nmol s⁻¹ μAmp⁻¹. Starting at 4 h, the laser was on a single colony for 1 h followed by 1 h of darkness before the next colony was irradiated. The inset shows expanded plots and integrated yields for each colony. Colonies were 14 d old.

Fig. 4. Longevity of hydrogen photoevolution in a single 14-d-old colony multiply irradiated at 1-h intervals. Points indicate integrated yields of hydrogen and are plotted at the beginning of the irradiation periods.

Fig. 5. Hydrogen yield-time profiles from nine individual colonies, each experiencing different dark times prior to the first irradiation. The yield of hydrogen from the first irradiation of each colony varies from the others by a factor less than twofold. First-irradiation yields were taken as 100%, with yields from subsequent irradiations being normalized to the first. Each individual colony is designated with a different symbol to allow the H₂ production of each colony to be followed for multiple irradiations.
stability of the colonies rather than a consequence of the dark time prior to the first irradiation.

**Colony Age, Chl Content, and Size.** The age of a colony, measured relative to the time of plating, has several effects on the apparent ability of a colony to produce H2. Figure 6 shows that colonies of different ages screened under the same conditions did not behave similarly. Fourteen-d-old colonies reproducibly gave the best response with regard to H2 per mass of Chl at multiple time points. Colonies much younger than 14-d-old (9 d in Fig. 6) gave high H2 to Chl yields at first but were much less stable over time than 14-d-old colonies. Older, larger colonies contained more Chl and occupied more surface area than younger colonies. Because of the area and density of older colonies, the laser beam was neither large enough to irradiate an entire colony nor intense enough to saturate photoevolution (Fig. 6). H2 evolution was light saturated in 14-d-old colonies as determined by comparing the total yields of H2 produced by laser irradiation and white light (Fig. 7).

**Colony Diameter and Chl Content.** Several 14-d-old colonies

![Fig. 6. Colony age and hydrogen photoevolution. Colonies were screened for 1 h followed by 1 h of darkness. Points are plotted at the beginning of the irradiation period. Chl content of the four colonies: 9 d old = 0.31 µg; 14 d old = 2.2 µg; 16 d old = 1.9 µg; and 21 d old = 3.2 µg. Repetitions of this experiment confirmed that 14-d-old colonies performed best and that only the early time points should be used to evaluate colonies. Photoevolution at late time points (after 24 h) varied widely in other experiments.](image)

were analyzed in order to determine Chl content and diameter. Results showed that colony size was roughly correlated with the amount of Chl per colony, and the total H2 yield generally increased with diameter. This response may be accounted for by a possible relationship between colony geometry and photoevolution. A colony that occupies more area may be less optically dense so that each cell will receive more light than would a compact, more dense colony. Therefore, increased total H2 yield in wider colonies may reflect the better light absorption configuration of the colonies.

Table II shows the total H2 yield, yield per mass of Chl, and Chl per colony for several 14-d-old colonies. The yield of H2 per unit Chl varied approximately inversely with Chl per colony.

**Survivability and Cloning of Screened Colonies.** In experiments where screened colonies were cloned following 24 h for anaerobiosis, they were excised from the agar and suspended in liquid minimal medium. Only 40% or less of the colonies typically survived screening (data not shown). There was no obvious correlation between colony survival and H2 production. In some cases, screened colonies were not excised from the plates but, instead, were placed back in the incubator. Most colonies turned white within 3 d of returning to standard growth conditions. The few surviving colonies were found to contain only a few surviving cells that grew as green microcolonies within the white (dead) parent colony. These survivors were propagated, and colonies were screened for anaerobic tolerance (i.e. increased survivability following screening) and H2 production. They showed no apparent differences when compared with unselected colonies (data not shown).

**Experimental Parameters.** Based on the data obtained from control experiments, only ~14-d-old colonies were used to examine variability among members of a population of algal cells. The unattenuated laser beam was used to irradiate the colonies, and the experiments were designed to screen all colonies within 24 h, with a maximum of 10 colonies being screened per experiment. Chl was extracted following screening, and H2 photoevolution by the colonies was compared on the basis of H2 per unit of Chl. Under these prescribed conditions, about 200 colonies were screened for inherent variability in H2 photoevolution. No colony was detected which had H2 yields greater than twofold more than the rest of the colonies screened during the same experiment (data not shown).

**DISCUSSION**

The results of these experiments demonstrate that photo-stimulated H2 evolution from single algal colonies can be measured. However, the yields were influenced by a number of key experimental parameters, the control of which was important for correct interpretation of the data. Colonies grown under the same conditions and on the same plate varied in H2 production by no more than twofold. Synchronously grown liquid cultures of *Scenedesmus obliquus* were shown by Senger and Bishop (19)

<table>
<thead>
<tr>
<th>Colony</th>
<th>Total H2 (nmol)</th>
<th>Chl/Colony (µg)</th>
<th>H2/Chl (nmol/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.2</td>
<td>1.10</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>0.78</td>
<td>24.4</td>
</tr>
<tr>
<td>3</td>
<td>21.6</td>
<td>0.95</td>
<td>23</td>
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<tr>
<td>4</td>
<td>23.5</td>
<td>1.26</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>25.5</td>
<td>0.97</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>26.2</td>
<td>1.45</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>25.5</td>
<td>1.62</td>
<td>15.9</td>
</tr>
<tr>
<td>8</td>
<td>25.7</td>
<td>1.44</td>
<td>17</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>1.01</td>
<td>20.8</td>
</tr>
<tr>
<td>10</td>
<td>15.4</td>
<td>0.99</td>
<td>15.5</td>
</tr>
</tbody>
</table>

![Fig. 7. Light saturation of 14-d-old colonies using white light. Seven colonies were irradiated with white light from a 300-W, ANSI code, ELH projector bulb. The colonies were irradiated for 1 h each with four different radiant fluxes, generated by varying the voltage to the bulb (40, 60, 70, and 80 V were used). The total yield of H2 at each radiant flux was compared with the summed yield of each colony irradiated with the laser for 1 h. The summed yield is represented by the arrow.](image)
to vary in H₂ photoproduction during their life cycle. C. reinhardtii growing as colonies did not display any life-cycle-dependent variation in H₂ production when synchronous and asynchronous (grown in constant light) colonies were compared. Therefore, the value of twofold was taken as the normal range of variability for C. reinhardtii under our experimental conditions.

The development of an apparatus with the sensitivity for detecting photogenerated H₂ from a single colony has a number of pertinent applications. At a rate of 10 colonies per day, the apparatus can be used to screen natural populations of algae for H₂ photoevolution. This approach for making putative identifying of interesting new strains is much more efficient than isolating pure cultures and screening them individually. Similarly, the apparatus can be used as a secondary screen to test putative mutants altered in their H₂ producing ability and hydrogenase. The simple addition of a wild-type colony to a plate of putative mutants will provide a powerful internal control for evaluating mutants. Since the general rule-of-thumb for algal mutants is that the phenotype should deviate from wildtype by 5-fold to 10-fold (14), the inherent variability of 2-fold indicated by this work will not interfere with mutant screening. The apparatus can also be used for comparing the overall response of algal colonies growing on semisolid medium to liquid cultures. Low post-screening viability mandates a colony replication scheme so that interesting colonies can be recovered.

An important addition to the apparatus would be an O₂ sensor of sufficient sensitivity to detect photoevolved O₂ (<10 nmol/h). A Hirsch galvanic cell (1, 11) was installed in the system, but the signal to noise ratio was too great to allow detection of O₂ evolved by a single 14-d-old colony. An O₂ luminometer (5) was another option, but its use required that the helium flow from the reaction chamber be split into two streams, one for the H₂ analyser and one for the luminometer. The gas analyzer is sensitive to dimethyl sulfoxide, the solvent used in the luminometer, and the luminometer cannot be placed downstream from the analyzer because of the O₂ flow that purges the analyzer. Splitting an already small signal was considered impractical, so the luminometer was not tested.

Even with the current inability to detect O₂, the apparatus described here is a powerful and quantitative tool which should find pertinent applications in a number of areas involving aerobic photosynthesis and H₂ photoevolution. It exceeds other detection methods in sensitivity and long-term stability and is uniquely suited for measuring steady state H₂ photoevolution for extended periods of time. Combining point irradiation, using a laser with the H₂ sensor, has produced a novel method for efficiently examining H₂ photoevolution from individual clones of an algal population.

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