A Simple Method To Demonstrate the Enzymatic Production of Hydrogen from Sugar

Natalie Hershlag
Syosset High School, Syosset, NY 11791

Ian Hurley
Department of Obstetrics and Gynecology, North Shore University Hospital, Manhasset, NY 11030

Jonathan Woodward*
Chemical Technology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6194

There is current interest in and concern for the development of environmentally friendly bioprocesses whereby biomass and the biodegradable content of municipal wastes can be converted to useful forms of energy. For example, cellulose, a glucose polymer that is the principal component of biomass and paper waste, can be enzymatically degraded to glucose, which can subsequently be converted by fermentation or further enzymatic reaction to fuels such as ethanol or hydrogen.

These products represent energy source alternatives to fossil fuels such as oil (*1, 2*). Demonstration of the relevant reactions in high-school and undergraduate college laboratories would have value not only in illustrating environmentally friendly biotechnology for the utilization of renewable energy sources, such as cellulosic wastes, but could also be used to teach the principles of enzyme-catalyzed reactions. In the experimental protocol described here, it has been demonstrated that the common sugar glucose can be used to produce hydrogen using two enzymes, glucose dehydrogenase and hydrogenase. No sophisticated or expensive hydrogen detection equipment is required—only a redox dye, benzyl viologen, which turns purple when it is reduced (*3*). The color can be detected by a simple colorimeter. Furthermore, it is shown that the renewable resource cellulose, in its soluble derivative form carboxymethylcellulose, and aspen-wood waste are also sources of hydrogen if the enzyme cellulase is included in the reaction mixture (*4*).

Background and Theory

The enzyme mixture called cellulase catalyzes the hydrolysis of cellulose to glucose. Three main types of activity—cellobiohydrolase, endoglucanase, and β-glucosidase—act synergistically to degrade cellulose to glucose (*5, 6*). Carboxymethylcellulose (CMC) is a soluble derivative of cellulose that is used as a bulking agent in foodstuffs and pharmaceuticals (*7*). It is formed in multiton quantities by the treatment of cellulose with sodium monochloroacetate under alkaline conditions.

Glucose dehydrogenase (GDH) is an enzyme that oxidizes glucose to gluconic acid. It requires a coenzyme, nicotinamide adenine dinucleotide phosphate ($NADP^+$), that accepts two electrons from glucose to become NADPH (*8*). Recently, it was found that NADPH, in an unusual reaction, could be oxidized directly by the enzyme hydrogenase, isolated from the deep-sea extremophile *Pyrococcus furiosus* (*9, 10*), with the evolution of hydrogen gas (*2*). It occurred to us that these

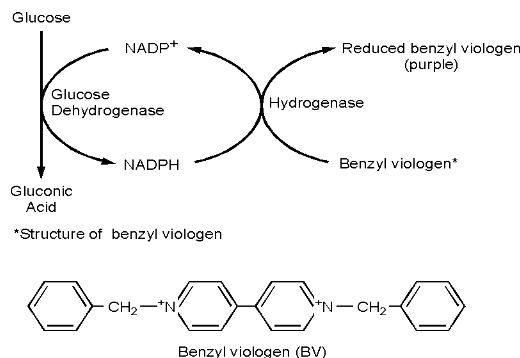

Figure 1. Reaction scheme for the measurement of hydrogen produced by the oxidation of glucose using benzyl viologen.

enzymatic reactions could be coupled to produce hydrogen from cellulosic materials and that such reactions would be appropriate for high-school class demonstrations as well as science projects and undergraduate laboratories, if hydrogen formation could be detected by a simple means (i.e., without the need for sophisticated equipment such as a gas chromatograph). By altering the reaction conditions, the principles governing enzyme reactions could also be illustrated (e.g., effects of pH, temperature, etc.). To detect hydrogen production, the redox dye benzyl viologen (BV) was used. This dye, which turns purple when reduced, is a relatively harmless chemical with a health hazard rating of only l. Its molar extinction coefficient is 7400 at 600 nm (*3*). Less than 1 μmol (10^{-6} mol) of reduced BV can be detected. The stoichiometry of the reaction is such that 1 mol of glucose reduces 1 mol of BV; therefore, by measuring the absorption of BV at 600 nm, the amount of hydrogen produced can be estimated. The overall reaction scheme is shown in Figure 1.

Experimental Procedure

These experiments were undertaken as an independent study during the spring term of the high-school sophomore year. Duplicating and extending this work should lie within the scope of laboratory activities for other high-school students as well as undergraduates.

This section summarizes the instructions and the questions given at the beginning of this project, including the basics needed to work effectively with minimal supervision in a particular research laboratory. These instructions will not suit all situations. For example, a colorimeter that can measure transmitted light near 340 and 600 nm may be substituted for a UV–vis spectrophotometer if the latter is not available.[1]

*Corresponding author.

Production of NADPH from Glucose

Unless otherwise stated, all reagents and enzymes and the coenzyme $NADP^+$ were obtained from the Sigma Chemical Company. Buffer solutions were made up in distilled water. The basic reaction mixture in a 1.0-mL volume of 50 mM sodium phosphate buffer, pH 8.0, consisted of $NADP^+$ (1.0 mM), glucose (10 mM), and diluted glucose dehydrogenase (from *Bacillus megaterium*) containing 0.17 unit of the manufacturer's stated activity (for details, see below). The increase in absorbance at 340 nm due to the formation of NADPH was monitored in a Shimadzu automatic recording UV–vis spectrophotometer, which was blanked with the reaction mixture minus the enzyme. One unit of activity is defined as the amount of enzyme that will oxidize 1 μmol of glucose per minute under the assay conditions.

Basic Laboratory 1

Demonstration of NADPH production is the best starting point for a study of these coupled enzymatic reactions. The laboratory techniques acquired at this stage will permit you to work independently.

Apparatus/Equipment Suggested

- Digital micropipettors, one adjustable from 0–200 μL, another from 0–20 μL
- Plastic culture tubes with screw caps: 50 mL and 15 mL, to store reagents
- Minifuge tubes: 1.7 ml for storage of enzymes
- Magnetic stirrer and stirring bars
- pH meter
- UV–vis spectrophotometer (340 and 600 nm)
- Spectrophotometer cells, 1.0-cm light path length, 1.0-mL total volume (plastic cells are satisfactory)
- Timer

Reagent Preparation

50 mM sodium phosphate buffer, pH 8.0. For 100 mL, dissolve 1.38 g of sodium dihydrogen phosphate in 50 mL of distilled water. Use a beaker, a stirring bar, and the magnetic stirrer. Use the pH meter to monitor pH as you stir. Prepare 1.0 M sodium hydroxide as described below. Add 1.0 M sodium hydroxide solution dropwise to the phosphate solution. Follow the pH change with the pH meter. Add the drops slowly as you approach pH 8, to allow time for the solutions to mix and react. Transfer all the buffer to a 100-mL measuring cylinder, add a stirring bar to the cylinder, and stir as you add reagent water to the 100-mL mark. The buffer should be stored in two 50-mL sealed culture tubes at room temperature. It should be discarded when cloudiness is observed or "white strings" develop in the tube.

1.0 M sodium hydroxide solution. CAUTION: Sodium hydroxide is corrosive and poisonous. In a 15-mL or larger polyethylene test tube, add four pellets of sodium hydroxide (~0.4 g) to 10 mL of water. Cap *very* securely and shake until dissolved. Be careful; the tube may become warm. Prepare this solution only as needed. It rapidly absorbs carbon dioxide from air.

10 mM $NADP^+$ solution. Dissolve 0.0765 g of nicotinamide adenine dinucleotide phosphate (Sigma Chemical Company) in 10 mL of reagent water. This solution should be "substocked" into ten 1.7-mL plastic minifuge tubes (Fisher) for storage in a freezer. Each day, thaw only the amount of $NADP^+$ you will use.

100 mM glucose. Dissolve 1.8 g of glucose in 100 mL of distilled water. Store in a refrigerator.

Glucose dehydrogenase. This is supplied as a solid in a bottle. Dissolve the contents in 1.5 mL of buffer, pH 8.0. Dilute 10 μL of this solution to 1.0 mL with 50 mM sodium phosphate buffer, pH 8.0, in a 1.7-mL minifuge tube. Store both the stock enzyme and the 1:100 dilution in a refrigerator.

Procedure

To two 1.0-mL spectrophotometer cells, add the following solutions in the order listed. Mix with your micropipet tip after each addition.

- 790 μL of 50 mM sodium phosphate buffer, pH 8.0
- 100 μL of 100 mM glucose
- 10 μL of 10 mM $NADP^+$ solution

Place one solution in the reference cell position in your spectrophotometer and the other in the sample cell position. Zero the absorbance reading at 340 nm. Add 100 μL of phosphate buffer to the reference cell and mix it thoroughly. Remove the cell from the sample cell position. Get your timer ready. The reference solution contains everything except GDH.

To Start the Reaction

Add 100 μL of the 1:100 diluted GDH to the cell and start your timer.

Place the cell back into the sample cell holder of the spectrophotometer and record the absorbance at 10- to 20-s intervals for at least 5 min, or until the absorbance stops changing.

Graph the absorbance as a function of time after the enzyme was added. The increase in absorbance is due to the NADPH formed in the reaction. The rate of the reaction is the slope of your graphed line.

Questions

- Is the rate of your reaction constant at first? Why or why not?
- Does the rate of your reaction ever become zero? If so, when? What has happened?
- What additional experiments could you do to find out how the rate of the initial reaction depends on the concentration of glucose in the cell? On the concentration of $NADP^+$?
- How could you find out if the rate of reaction depends on the concentration of enzyme in the cell? On temperature? On added salt (sodium chloride)? On pH? Try out some of your ideas about experiments. Explain what you discover using your knowledge of chemical kinetics.

Production of Hydrogen from Glucose

For the production of hydrogen from glucose, the basic reaction mixture was as described above but also included partially purified *Pyrococcus furiosus* hydrogenase, containing 5 units of the manufacturer's (University of Georgia) stated activity plus BV (4.0 mM stock in buffer pH 8.0) in the 1.0-mL reaction volume (for details, see below). The increase in absorbance was monitored at 600 nm. The utilization of BV, we reasoned, would be an appropriate alternative for detecting the formation of hydrogen without the use of sophisticated gas chromatography or other techniques normally needed.

Basic Laboratory 2

Partially purified *Pyrococcus furiosus* hydrogenase transfers hydrogen from NADPH (produced by the reaction described above) to BV, the hydrogen detector. Set the spectrophotometer to 600 nm to monitor the concentration of the reduced form of BV.

Reagents and Enzymes

40 mM BV. Dissolve 0.164 g of BV in 10 mL of sodium phosphate buffer, pH 8.0.

Pyrococcus furiosus hydrogenase is used without further dilution and is provided in an air-free vial sealed with a rubber septum (through which a syringe needle will pass). This enzyme is sensitive to air, and care should be taken not to expose it to air prior to its use.

Procedure

Prepare reference and sample cell solutions in 1.0-mL cells by adding these solutions in the following order, mixing between additions:

660 µL of 50 mM sodium phosphate buffer, pH 8.0
100 µL of 100 mM glucose
100 µL of 40 mM BV

Then add 140 µL of the buffer to the reference cell to bring its volume to 1.00 mL, and use this solution to zero the spectrophotometer at 600 nm.

Now add to the sample cell 100 µL of 1/100 diluted GDH and 30 µL of *Pyrococcus furiosus* hydrogenase.

Record your observations.

Initiate the reaction by adding 10 µL of 10 mM $NADP^+$ solution to the sample cell. Start the timer, and add the coupling reagent at the same time; place the sample cell in the spectrophotometer immediately. The reference sample contains everything except $NADP^+$.

Record the absorbance (600 nm) as a function of time, and graph it as before.

Questions

How does the initial rate of this reaction differ from the initial rate of the first reaction studied?

How does the maximum rate of this reaction compare with the maximum rate of the first reaction? (HINT: How can you correct for the different intensities of the absorbances of NADPH and reduced BV?)

Would reduced BV be made if no hydrogenase were present?

Does the maximum rate of this reaction change if you change the concentration of $NADP^+$ in the sample cell? The concentration of glucose? The concentration of glucose dehydrogenase?

Try out some of your ideas. Explain your findings in terms of your knowledge of chemical kinetics.

Production of Hydrogen from Cellulose

A 4% solution of CMC was dissolved in the buffer solution, pH 8.0. A commercial cellulase preparation (Celluclast) was a gift from Novo Nordisk Bioindustrials and was subjected to gel filtration on a Sephadex G-25 (PD-10) column (from Pharmacia) equilibrated in buffer, pH 8.0. It had a protein concentration of 20 mg/mL. The reaction mixture (1.0 mL) consisted of CMC, cellulase, GDH, hydrogenase, $NADP^+$, BV, and buffer, pH 8.0 (for details, see below). The absorbance at 600 nm was monitored over time.

Basic Laboratory 3

Finally, we add the initial reaction to the coupled reaction scheme. CMC, a soluble cellulose, is converted to glucose by a mixture of enzymes referred to as cellulase.

Reagents and Enzymes

4% (w/v) CMC. Dissolve 1 g of CMC in 25 mL of sodium phosphate buffer, pH 8.0.

Cellulase. Celluclast, a product of Novo Nordisk Bioindustrials, needs to be filtered initially to remove soluble sugar in the crude enzyme preparation. This is achieved by gel filtration using a disposable column of Sephadex G25 (Pharmacia) equilibrated in the sodium phosphate buffer.

Procedure

Prepare reference and sample cell solutions in 1.0-mL cells by adding these solutions in the following order, mixing between additions:

250 µL CMC
100 µL of 40 mM BV
460 µL of buffer, pH 8.0

Zero the spectrophotometer at 600 nm as before, after adding 190 µL of buffer to the reference cell to bring its volume to 1.0 mL. From this point on, observe carefully and record your observations.

Then add to the sample cell 100 µL of 1/100 diluted GDH and 30 µL of *Pyrococcus furiosus* hydrogenase, followed by 10 µL of 10 mM $NADP^+$ solution.

Finally, start the reaction (and the timer) by adding 50 µL of cellulase.

Place the cell in the spectrophotometer immediately and monitor; then plot the absorbance at 600 nm. The reference sample contained everything except cellulase.

Questions

How do the kinetics of the this reaction differ from your previous results?

Would predigesting the CMC with cellulase change your results? Can you devise an experiment to test your predictions?

Results and Discussion

The data of Figure 2 show that glucose was oxidized by GDH in the presence of $NADP^+$, resulting in an increase in absorbance at 340 nm due to the formation of NADPH. The reaction rate was linear for the first 2 min, providing data from which the activity of the enzyme could be calculated (see below).[2] The GDH used in these experiments is known to be stabilized by high concentrations of sodium chloride (*11*). The presence of 3 M NaCl, however, had no effect on the rate of NADPH production, suggesting that GDH was stable without salt during the course of the assay.

It is known that the rate of an enzyme-catalyzed reaction is affected by different assay conditions. The effects of glucose and GDH concentration on the rate of NADPH formation are summarized in Table 1. The known molar extinction coefficient of NADPH (i.e., the absorbance of a 1 M solution at 340 nm is 6220) allows the calculation of NADPH concentration formed during the assay. These data illustrate two important principles of enzyme-catalyzed reactions: (i) the rate of the reaction increases linearly as the enzyme concentration increases; and (ii), the rate is also dependent on

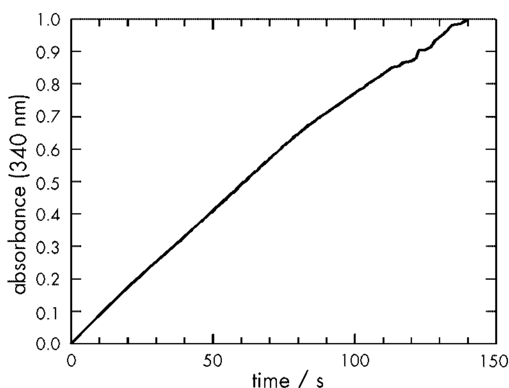

Figure 2. Oxidation of glucose by glucose dehydrogenase: production of NADPH. The reaction mixture contained glucose (10 μmol), glucose dehydrogenase (0.17 unit), and $NADP^+$ (1 μmol) in 1.0 mL of 50 mM sodium phosphate buffer, pH 8.0, at room temperature (25 °C). NADPH formation was monitored by the increased absorption at 340 nm.

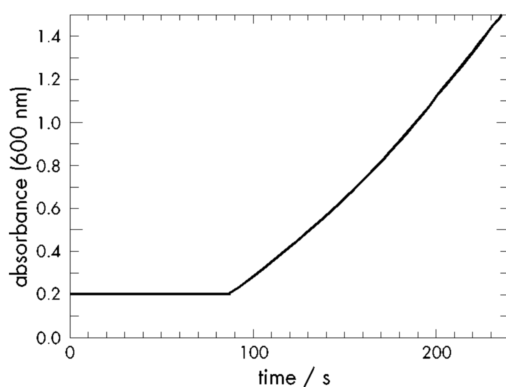

Figure 3. Production of hydrogen from glucose by glucose dehydrogenase and hydrogenase. The reaction mixture was as described in the legend for Fig. 2 but also contained 10 μL of hydrogenase (5 units) and 0.1 mL of benzyl viologen (4 mM final concentration). The formation of hydrogen was monitored by the increase in absorbance at 600 nm due to the reduction of benzyl viologen. The shift in baseline is insignificant and does not affect reaction rate determination.

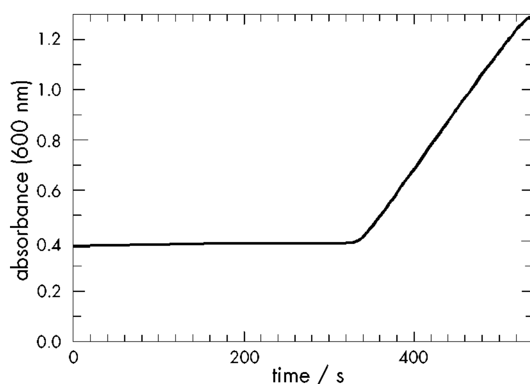

Figure 4. Production of hydrogen from carboxymethylcellulose. The reaction mixture was as described in the legend for Fig. 3 but also contained 1% (w/v) carboxymethylcellulose (medium viscosity) and 0.05 mL of cellulase (1.0 mg of protein). The shift in baseline is insignificant and does not affect reaction rate determination.

Table 1. Effect of Glucose and Glucose Dehydrogenase Concentration on Rate of NADPH Formation

Glucose concn/mM	Glucose Dehydrogenase (manufacturer's units)	NADPH Formation/ μmol min^{-1}
1.0	0.17	0.010
10.0	0.17	0.073
100.0	0.17	0.068
10.0	0.42	0.017
10.0	0.85	0.034
10.0	1.7	0.097

NOTE: For other conditions, see legend for Fig. 2.

substrate concentration. There was no increase in the rate of NADPH formation when the glucose concentration in the assay was increased from 10 to 100 mM. Between these concentrations the available enzyme was, presumably, working at its maximum rate. It should be noted that the reason for the lower rate of formation of NADPH over time, observed during the experiments carried out on the effect of enzyme concentration, was due to the loss of GDH activity during storage without NaCl. This system could also be used to easily illustrate the effects of other parameters (e.g., pH, temperature) on the rate of this enzyme-catalyzed reaction.

The reaction mixture containing glucose, GDH, BV, and hydrogenase in buffer pH 8.0 initially turned purple owing to the formation of some reduced BV but rapidly oxidized in air, becoming colorless. This color change was caused by residual sodium dithionite in the hydrogenase preparation that reduced BV. When $NADP^+$ was added to the reaction mixture, there was a linear increase in absorbance at 600 nm due to the formation of reduced BV (Fig. 3). These data are noteworthy because they show that BV can be used to detect hydrogen produced by the coupling of GDH and hydrogenase using glucose as the substrate. Also, the production of hydrogen was observed approximately 1.5 min after the addition of $NADP^+$, suggesting that a threshold level of reductant was necessary prior to the reduction of BV catalyzed by hydrogenase. After 3 min of the reaction, it was determined that 0.11 μmol of reduced BV was formed. This would correspond to the concentration of molecular hydrogen generated if protons, instead of BV, were used as the electron acceptor in the reaction. In a control experiment, it was established that hydrogenase was essential for the reduction of BV.[3]

The data of Figure 4 show that cellulose can be converted into hydrogen if the enzyme cellulase is included in the reaction mixture. These are the first data to show that CMC (an abundant and soluble cellulose derivative) can be used to generate hydrogen fuel. A lag time of almost 6 min occurred before the onset of the reduction of BV. This longer lag time, compared with that observed when starting with glucose, could be explained by the need for CMC to be hydrolyzed to glucose prior to oxidation of the latter followed by reduction of BV.[4]

The current interest in the relationship between greenhouse gases and global warming is also relevant to the work described in this paper. One way to reduce the potential harmful effects of greenhouse gases is to use renewable energy resources rather than fossil fuels. Thus waste cellulosic materials offer a major source of hydrogen because their major component is sugar.

On an annual basis, there is more wastepaper and wood waste generated than the timber harvested in the United States of America (*12*). We have demonstrated that aspenwood waste could be used as a source of hydrogen. Hydrogen was evolved when it was incubated with cellulase, glucose dehydrogenase, $NADP^+$, and hydrogenase in buffer solution, pH 7.5 at 50 °C (Fig. 5). In this experiment hydrogen gas was actually measured using a tin oxide semiconductor (*2*). If benzyl viologen had been incubated with the reaction mixture it would have been reduced as shown in the experiments described above. These data show that the maximum rate of hydrogen production reached was approximately 4.6 µmol/h. In terms of yield, when the experiment was stopped after about 16 h, 56 µmol of hydrogen was evolved. The main point of these data is that any cellulosic waste could be used as a source of hydrogen and this would be demonstrable in the classroom setting using the techniques described in this work, for example, the conversion of CMC to hydrogen.

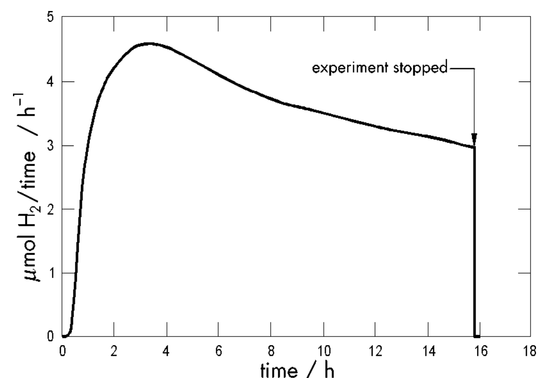

Figure 5. Production of hydrogen from aspen wood. The reaction mixture (2.0 mL) contained 10 mg of aspen wood, 12.5 units of GDH, 50 units of hydrogenase, and 0.5 mL of cellulase in 50 mM sodium phosphate buffer, pH 7.5, at 50 °C. The reaction was started by adding $NADP^+$ (1 µmol). For details of the experiment apparatus and method for hydrogen detection, see ref *2*.

Conclusions

These experiments offer a simple method for illustrating the principles of enzyme-catalyzed reactions as well as the bioconversion of renewable resources to energy (in the form of molecular hydrogen) for students in high-school and undergraduate laboratory courses, research projects, and demonstrations. They also introduce students to the application of enzymes and the utilization of nature's catalysts in environmentally friendly biotechnology—in this case for the production of hydrogen gas, which could become the fuel of the 21st century.

Notes

1. A simple colorimeter without UV capabilities can be used if a UV–vis spectrophotometer is not available for hydrogen measurements.
2. Calculation of enzyme activity of GDH. From Fig. 2, we can calculate the rate at which NADPH is formed in units of µmol min^{-1} mL^{-1} undiluted enzyme. This corresponds to units of activity per milliliter of enzyme.
3. Other redox dyes (i.e., reactive triazine dyes) might also accept electrons from NADPH and change color.
4. It would be easy to demonstrate this reaction starting with other sugars such as starch, sucrose, and lactose if the enzymes amyloglucosidase, invertase, and lactase, respectively, were included in the reaction mixture to break down these substrates with resulting glucose production.

Acknowledgments

We gratefully acknowledge Susan Benoff of North Shore University Hospital, Manhasset, NY, for her support and guidance during the course of this project. We also thank Laura Wagner for administrative support and Martha Stewart for editorial assistance. This work was also supported by the Chemical Sciences Division, Office of Basic Energy Sciences, U.S. Department of Energy, under contract DE-AC05-96OR22464 with Lockheed Martin Energy Research Corp., and by the Christopher Columbus Fellowship Foundation.

Literature Cited

1. *Fuels and Chemicals from Biomass*; Saha, B.; Woodward, J., Eds.; ACS Symposium Series 666; American Chemical Society: Washington, DC, 1997.
2. Woodward, J.; Mattingly, S. M.; Danson, M. J.; Hough, D. W.; Ward, N.; Adams, M. W. W. *Nature Biotechnol.* **1996,** *14*, 872–874.
3. Ballantine, S. P.; Boxer, D. H. *J. Bacteriol.* **1985,** *163*, 454–459.
4. Woodward, J. In *Carbon Substrates in Biotechnology*; Stowell, J. D.; Beardsmore, A. J.; Keevil, C. W.; Woodward, J. R., Eds.; IRL: Oxford, 1987; p 45.
5. Goyal, A.; Ghosh, B.; Eveleigh, D. *Bioresource Technol.* **1991,** *36*, 37–50.
6. Woodward, J. *Bioresource Technol.* **1991,** *36*, 67–75.
7. *The Merck Index*, 9th ed.; Windholz, M., Ed.; Merck and Co.: Rahway, N J, 1976.
8. Budgen, N.; Danson, M. J. *FEBS Lett.* **1986,** *196*, 207–210.
9. Bryant, F. O.; Adams, M. W. W. *J. Biol. Chem.* **1989,** *264*, 5070–5079.
10. Ma, K.; Hao Zhou, Z.; Adams, M. W. W. *FEMS Microbiol. Lett.* **1994,** *122*, 245–250.
11. Pauly, H. E.; Pfleiderer, G. *Biochemistry* **1997,** *16*, 4599–4604.
12. Woodward, J.; Stephan, L. M.; Koran, L. J., Jr.; Wong, K. K. Y.; Saddler, J. N. *Bio/Technology* **1994,** *12*, 905–908.