The Biosphere: Problems and Solutions, edited by T.N. Veziroğlu
Elsevier Science Publishers B.V., Amsterdam, 1984 — Printed in The Netherlands

HYDROGEN PRODUCTION BY NON-PHOTOSYNTHETIC BACTERIA

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ABSTRACT

This paper is devoted to the identification of hydrogen producing non-photosynthetic bacteria and the discussion of the following three areas of possible research: 1) hydrogen from sewage treatment plants; 2) hydrogen from rumen bacteria; and large scale production of hydrogen through the genetic manipuation of hydrogen producing non-photosynthetic bacteria.

KEYWORDS

Hydrogen production; non-photosynthetic bacteria; sewage treatment plant; rumen bacteria; hydrognease; formate dehyrogenase.

INTRODUCTION

International problems with air pollution (acid rain, health effects, property damage, etc.) have raised public awareness to the need for a non-polluting energy source. Hydrogen has, in the past, been suggested as a fuel which would eliminate most air pollution problems. One reason for the delayed acceptance of hydrogen has been the difficulty of production on a cost-effective basis. One method of hydrogen production with great promise has been that by unicellular organism synthesis. Photosynthetic and non-photosynthetic organisms have been studied and suggested as possibilities. Extensive analytic reviews of algae and photosynthetic microorganisms have been published. However, photosynethic organisms require solar collectors, and engineering analysis has suggested that solar generators would be too costly. Therefore this paper suggests that the use of non-photosynthetic bacteria would be more cost effective in producing hydrogen. Bacterial classification, enzyme identification and future large scale production designs will also be addressed.

BACTERIAL PRODUCTION OF HYDROGEN

Historically, science has known about hydrogen production from micro-organisms for over fifty years, but most of the research has been conducted with photosynthetic organism. Weaver (1980) at Solar Energy Research Institute (SERI), and Mitsui (1980) at the University of Miami have led the experimental work in the fields of photobiosynthetic bacteria and algae. A brief examination of the practicability of establishing a generator utilizing cultures of Rhodopseudomonas capsulata led us to the conclusion that long term efficiency would not be cost-effective. Data supplied by (SERI) were analyzed by Lummus/Combustion Engineering Corp. of Englewood, New Jersey. Lummus (1981) concluded that with a 5% efficiency, the payback time would be about 11.5 years.

With this information we were forced to seek out other options for hydrogen production, specifically non-photosynthetic microorganisms for the production of hydrogen. Reviewing the literature on this subject, we found evidence of hydrogen production in humans.

CLINICAL EVIDENCES OF HYDROGEN PRODUCTION IN MAN

Man has known about explosive intestinal gases for centuries. It has been suggested that ancient Chinese Dragons eructed hydrogen and methane which ignited to form the historical "flaming breath." Levy (1954) reported evidence of an explosive mixture of intestinal gases (IG) found during electro-surgery. Bigard (1979) also reported the occurrence of a fatal explosion during colonic polypectomy.

Analysis of this explosive IG was reported by Levitt (1970). He found that hydrogen could reach as high as 45% of the total IG of the lower intestine. LaBrooy (1981) found that hydrogen concentration increased when mannitol was used as a pre-operative laxative. When the hydrogen levels reach 4.1% it is considered explosive. A level of 45% was therefore a life-threatening situation. Further analysis of IG by Perman (1981) utilizing gas chromatography and mass spectrometry concluded that hydrogen production rates were pH dependent, with an optimum range from 5.5 to 7.6, with maximum production at 7.0 to 7.4.

Additional information regarding hydrogen production was supplied by Levitt (1969) who found that hydrogen concentration increased following meals of non-absorbable carbohydrates. Perman measured glucose as the substrate, and concluded that bacteria can utilize glucose via pyruvate degradation. As long as glucose is available, hydrogen production was constant at 16.3 micromoles per hour at pH 7.3. Raskin (1980) also found that certain foods such as beans, raisins, bananas, fruit juices, and the antibiotic succinylsulfathiazole increased hydrogen production.

Since hydrogen is now known to be produced in the human intestine, we would expect that hydrogen could be obtained from human sewage. We will deal with this potential later. First, we must identify and classify these non-photosynthetic microorganisms and their mechanism of hydrogen production.

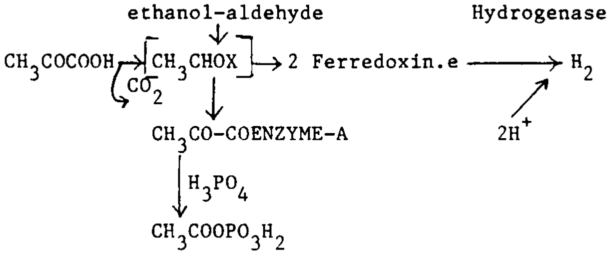
CLASSIFICATION OF ALL HYDROGEN PRODUCING ORGANISMS

Gray and Gest in (1965) wrote an excellent review on the classification of hydrogen producing organisms. They divided all the hydrogen producing organisms into the following four categories.

CATEGORY I

Strict anaerobes with hydrogenase enzyme and ferredoxin (which mediates the transfer of electrons to the enzyme) comprise Category I. The source of electrons are from any reduced two-carbon compounds (such as degraded pyruvate) products from purine and pyrimidine metabolism, and anaerobic degradation of amino acids.

Phosphoclastic cleavage of pyruvate with no formic acid intermediate appeared to be the mechanism for organisms in Category I. The electron carrier is a ferredoxin protein and not cytochrome. The following scheme represents the reaction of pyruvate degradation in Clostridium pasteurianum.



(1)

In this model Gray and Gest (1965) described how reduced two-carbon compounds, such as ethanol or aldehyde could enter the reaction and result in the formation of a CH_3CHO-X complex. Electrons are then passed onto the ferredoxin protein, where they eventually unite with two hydrogen ions to form molecular hydrogen (catalyzed by hydrogenase). The CH_3CHO-X can combine with Co-Enzyme A to form acetyl-Co-Enzyme. The presence of oxygen severely inhibits the generation of hydrogen, which could be interrupted as interference or competition by oxygen with hydrogen as the terminal electron acceptor.

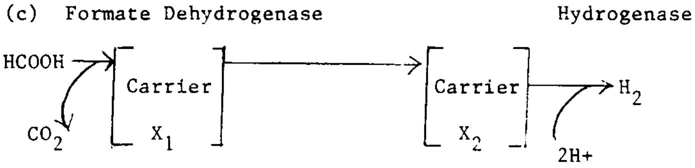
CATEGORY II

Facultative anaerobes that cleave pyruvate, forming a formic acid intermediate, are classed as Category II. The formic acid is produced through a phosphoclastic reaction catalyzed by a complex of soluble formic dehydrogenase. This enzyme coupled to an insoluble hydrogenase enzyme, along with two intermediate electron carriers are designated as X_1 and X_2. The following model shows the cleavage of pyruvate with formic acid as the intermediate.

(a) $CH_3COCOOH+H_3PO_4 \longrightarrow CH_3COOPO_3H_2+HCOOH$ (2)

(b) $HCOOH \longrightarrow CO_2+H_2$

(c)



Pyruvate is degraded through a phosphoclastic reaction to form formic acid(a). Formic acid is then degraded further into CO_2 and molecular hydrogen(b). Details of formic acid degradation (b) are shown in step(c). Electrons from formic acid are passed into X_1, which is identified as cytochrome reductase; while X_2 is identified as cytochrome C_{552} by Wimpenny

(1963), and Williams (1964). Finally the electrons are passed on to the hydrogenase enzyme for the formation of molecular hydrogen.

Facultative and heterotropic anaerobes such as E. coli are examples of the microorganisms found in category II. During anaerobic growth most of these bacteria can produce cytochrome reductase and cytochrome, which are the X_1 and X_2 electron carriers previously described.

Category I organisms utilitizes direct cleavage of pyruvate by the hydrogenase enzyme coupled to ferredoxin. In Category II, formate is oxidized to CO_2 and H_2 by formate hydrogenlyase which is a complex of formate dehydrogenase and hydrogenase enyzmes.

The enzyme systems of categories I and II are not the same. When formate dehydrogenase enzyme from E. coli (Category II) was mixed with the hydrogenase enzyme from C. pasteurianum (Category I) no hydrogen was produced. This evidence supports the obligatory association between formate dehydrogenase and hydrogenase enzymes in the Category II systems.

CATEGORY III

Only one microorganism is classified in Category III because of its unique characteristics. Desulfovibrio desulfuricans is a heterotrophic strict anaerobe with a cytochrome system of low redox potential (E_o = -205 mv). It can also catalyze the evolution of hydrogen from $Na_2S_2O_2$ in the presence of cytochrome C_3 or methyl viologene. The reaction however, does not take place in the presence of ferredoxin or methylene blue, which would imply an electron transfer mechanism similar to Category II. The hydrogenase enzyme is more like a Category I type enzyme. D.desulfuricans can also use sulfate, as will as hydrogen, as the terminal oxident. The actual process of pyruvate degradation produces a formic acid intermediate, like the mechanism for Category II. Gest suggested that the enzymes found in D.desulfuricans might represent a transition state with the "coli" type hydrogenase enzyme, which has a phosphoclastic type pyruvate breakdown found in Category I.

CATEGORY IV

All photosynthetic microorganisms are placed category IV, which consists of photosynthetic sulfur bacteria; photosynthetic non-sulfur purple bacteria; and the anaerobically adapted algae.

Gray and Gest determined that the function(s) of hydrogen evolution for each of the four categories. Using Clostridium pasteurianum as the example of a strict anaerobe in Category I, they proposed that hydrogen evolution was used for the disposal of electrons from energy-yielding oxidations processes. Using E. coli as the example of a facultative anaerobe in Categoy II, they speculated that hydrogen production promoted energy yielding oxidation through the removal of formate.

Desulfovibrio desulfuricans, the only organism in Category III, shares both the functions of Categories I and II. Lastly, in photosynthetic organisms (Category IV), hydrogen evolution is used for regulation of the reducing power and the energy pool. Readers are recommended to read this excellent article by Gray and Gest (1965).

PHOTOSYNTHETIC PRODUCTION OF HYDROGEN

A brief discussion of photobiological production of hydrogen will be summarized in order to have a better understanding of hydrogen production, but readers are recommended to read the review article by Dr. Paul Weaver.

When reviewing the history of hydrogen metabolism in microorganisms one can see the simulateous discovery of hydrogen uptake and hydrogen evolution, but under different environmental conditions. Photoautotrophic growth using hydrogen was first reported by Roelèsen (1934) in Chromatium vinosum, grown under light conditions. Three years later, hydrogen evolution was discovered in the same organism by Nakamura (1937) but the organism was grown in a dark environment. Both hydrogen uptake and evolution were reported in Rhodospirillum rubrum, grown under different environmental conditions (Gest 1949a, Gest 1949b, Kamen 1949). Hydrogen evolution in R .rubrum occurred when the organism was grown photosynthetically in media containing amino acids that served as the sole source of nitrogen, but hydrogen production ceased when nitrogen gas or ammonia ions were introduced into the culture. Conversely, Ormerod and Gest (1962) reported the autotrophic growth of R. rubrum utilizing hydrogen. It is concluded that almost all photosynthetic microorganism can produce hydrogen under certain environmental conditions.

HYDROGEN METABOLISM

Hydrogen metabolism can be expressed through the following equation:

$$H_2 \rightleftarrows 2H^+ \quad + \quad 2e \qquad (3)$$

Because this is a reversible reaction, the term hydrogen uptake is used to describe the ionization of molecular hydrogen (reaction from left to right); while the formation of molecular hydrogen (reaction from right to left) is referred to as hydrogen evolution. Ionization of molecular hydrogen is perhaps more common and can be found in many biochemical pathways, where the ionized hydrogen and its electron are carried through the electron transport system by Nicotine Adenine Dinucleotide(NAD); and the various cytochromes; eventually combining with molecular oxygen to form water, and ATPs. Hydrogen ions are utilized by aerobic organisms to make ATPs, through the electron transport systems, but what advantage would hydrogen evolution offer the organisms? Gray and Gest provided this following summary for hydrogen evolution in microorganisms. In the absence of an external electron acceptor (molecular oxygen), where the supply of energy is limited, some anaerobes have adapted to use inorganic compounds such as sulfate and nitrates as their terminal oxidants. Strict and facultative anaerobes use hydrogen as the terminal electron acceptor. A different reason is given for photosynthetic organisms that produce hydrogen. The energy supply and reducing power can accumulate and be in excess in relationship to the metabolic scheme of the entire organisms, thus hydrogen evolution in photosynthetic organisms is strictly for the elimination of excess electrons. In non-photosynthetic organisms, the hydrogen is serving as a terminal oxident electron acceptor during anaerobic growth conditions.

FUTURE POTENTIAL FOR NON-PHOTOSYNTHETIC BACTERIA HYDROG PRODUCTION

Since it is well established that hydrogen can be produced by non-photosynthetic bacteria, we must now speculate future possibility for large scale application. The abundance of hydrogen producing non-photosynthetic bacteria and the ease of growing these organisms would make them a preferred choice over photosynthetic hydrogen producers. The question remains, what are the options available for future research? It would seem logical that the source of hydrogen should be obtained from non-photosynthetic organisms already found in nature, such as in sewage treatment plants, or in animal waste. Still another approach would be to use pure cultures of genetically enchanced hydrogen producing non-photosynthetic microorganism on an industrial scale.

The remaining portion of this paper is devoted to the following three areas of possible research: 1) hydrogen from sewage treatment plants; 2) hydrogen from rumen bacteria; and 3) large scale production of hydrogen through the genetic manipulation of hydrogen producing nonphotosynthetic bacteria.

HYDROGEN FROM SEWAGE TREATMENT PLANTS

Hydrogen producing microorganisms from the Moorhead Minnesota sewage treatment plant were isolated and identified by Holmes (1978). Hydrogen producing organisms from the sewage sludge digester were isolated and grown in defined media, in which 84% of the tested organisms showed a preference for glucose. Enterobacteriaceae, consisting of genera Citrobacter(58%); Enterobacter(28%); and Escherichia (0.3%) were the hydrogen producers found in the digester. No obligatory anaerobes were identified as hydrogen producers in the sewage sludge. Although the major hydrogen producing organisms were not clearly identified, the Enterobacteriaceas were present in the highest concentration (1.4×10^7/ml) in sewage sludge. Lutgen (1982) observed hydrogen production in *Citrobacter freundii*, using a continuous culture system.

Obviously much more research is needed in the following areas: 1) identification of the specific species of hydrogen producers; 2) quantitation of the hydrogen yield, under optimum pH and temperature; 3) media specificity; and 4) optimum physical and chemical environment to enchance hydrogen yield.

We suggest that, since hydrogen is produced in the digester, the gases could be collected and the hydrogen could be separated by a molecular sieve or a prism premeable membrane to give us the purified gas as described by Moore (1983). It is conceivable that such a separation unit could be mounted on the existing sewage digester to collect hydrogen. If sufficient hydrogen could be collected, it would be the most economical means of producing hydrogen since sewage disposal is a growing problem with all city municipalities. The production and the sale of hydrogen could potentially provide the city with a revenue to off-set the cost of operating the sewage treatment plant.

HYDROGEN PRODUCTION BY RUMEN BACTERIA

Many species of hydrogen producing rumen bacteria have been reported. Joyner (1977) studied the following rumen bacteria in pure cultures and cell-free systems: *Bacteroides clostridiiformis*; *Butrivibrio fibrisolvens*; *Eubacterium limosum*; *Fusobacterium necrophorum*; *Ruminococcus albus*; and *Ruminococcus flavefaciens*. In all the preceding species the reaction appeared to be catalyzed by a soluble hydrogenase enzyme. The hydrogen was produced from the oxidation of pyruvate, but in *E.limosum*. *F. necrophorum*, and *B.clostridiformis* the hydrogen was associated with formate degradation. This would suggest the presence of a formate dehydrogenase enzyme system. When the hydrogenase enzyme was subjected to DEAE-cellulase treatment first, the hydrogenase activity was greatly inhibited. Joyner suggested that such an inhibitory reaction would imply the involvement of a ferredoxin-like electron carrier in these reactions.

Wolin (1980) studied the inhibitory affects of several, agents on hydrogen production in *R.albus*, *R.flavefaciens*, *R.bromei*, *B.fibrisolven*, and *Veillonella alcaleseens*. A complex of molybdate and sulfide at a concentration of 10^{-5}M inhibited hydrogen production, but not when molybdate or sulfide were placed into the growth media individually. The observed hydrogen inhibition was coupled with an increase of formate production. The data has led Wolin to suggest that a molybdate-sulfide complex inhibited the hydrogenase and diverted the electrons that were normally used to form molecular hydrogen to be used in the reduction of CO_2 to form instead of formate. The mechanism of hydrogen production in Rumen is not clear. *E.limosum*, *F.necrophorum*, *B. clostridiiformis*, *R. flavefaciens* produces hydrogen through a formate intermediate, which would suggest the presence of a formate hydrogenlyase complex. Joyner (1977) reported that the hydrogenase enzyme appeared to be soluble in all the organisms examined except *R. albus*, which did not form a formate intermediate to produce hydrogen.

Scheifinger (1975) reported an interesting finding in *Selenomonas ruminantium* grown with *Methanobacillus omelianskii*. Hydrogen was produced only when the *S. ruminanthium* was mixed with the methanogenic bacteria. Hydrogen evolution ceased when the *S. ruminantium* was grown independently in culture. The mechanism of hydrogen evolution was from reduced NADH formed during the degradation of glucose, glycerol or lactate. This mechanism is unlike any of the previously described mechanisms for hydrogen production.

It is clear that many rumen microorganisms are capable of producing hydrogen, and since many of these organisms have been isolated and identified, it is quite possible to employ a continuous culture fermentation processes for hydrogen production.

ENHANCEMENT OF HYDROGEN PRODUCTION THROUGH GENETIC ENGINEERING

It is interesting to find *E. coli* listed as one of the hydrogen producers in Category II. Although the hydrogen yield by *E. coli* may not be high, as Holmes (1978) indicated by the percentage distribution found in sewage sludge, *E. coli* is still best suited for genetic manipulations. It is quite possible to insert the genes responsible for hydrogen evolution into coli plasmids to enchance its hydrogen production. Of course, the nucleotide sequence of the enzymes responsible for hydrogen production must be identified and synthesized. This may be a monumental task.

Another approach would be to promote the growth of organisms with high hydrogen yield through genetic selections. However, direct genetic recombinant manipulation to increase hydrogen evolution is still more desirable. Presently hydrogenase and formate dehydrogenase are the only two enzymes for certain in hydrogen producing non-photosynthetic bacteria.

HYDROGENEASE AND FORMATE DEHYDROGENASE

The amount of information available on hydrogenase (EC-1.12.1.1 H_2:Ferredoxin Oxidoreductase) and formate dehydrogenase (EC -1.2.2.1-Formate:Cytochrome b_1 Oxidoreductase) are too voluminous to review in this article. Readers are recommended to read the following references for additional information on these two enzyme systems: Gest (1952); Peck (1957_a); Mortenson (1974); Peck (1957_b): Scherer (1978, 1982); Wagner (1977); Leonhardt (1977); Enoch (1982) and Hou (1982).

FUTURE POTENTIAL FOR NON-PHOTOSYNTHETIC HYDROGEN PRODUCTION

Ideally, large scale fermentation of genetically engineered microorganisms would be employed to produce maximum yield of hydrogen. Such a goal would require years of research and development, and would be very costly. We maintain that the most economically efficient approach would be to collect hydrogen from sewage treatment plant digesters, which in essence are already a large fermenters producing hydrogen. We propose the development of collecting hydrogen from sewage treatment plants in the following manner: 1) monitor the amount of hydrogen produced in existing sewage digesters to obtain a baseline level for hydrogen yield on a daily bases; 2) collect the effluent gases from the sewage digester through the uses of molecular sieves or prism permeable membrances separating hydrogen from the other gases; and 3) provide a storage system for the collected hydrogen gas. This approach would also facilitate the collection and the sale of methane gas which is another by-product from sewage digesters.

It is our contention that hydrogen producing non-photosynthetic bacteria should be considered as a source of hydrogen producer. Suggestions for future research should address the following questions:

1. Can present sewage digesters be converted to produce hydrogen on a large scale?
2. Can hydrogen be obtained from animal waste, or can hydrogen be obtained from rumen bacteria in controlled fermentation processes?
3. Can genetic engineering produce a more efficient hydrogen producing organism?

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