Microbial Production of Hydrogen: An Overview

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ABSTRACT: Production of hydrogen by anaerobes, facultative anaerobes, aerobes, methylotrophs, and photosynthetic bacteria is possible. Anaerobic *Clostridia* are potential producers and immobilized *C. butyricum* produces 2 mol H_2/mol glucose at 50% efficiency. Spontaneous production of H_2 from formate and glucose by immobilized *Escherichia coli* showed 100% and 60% efficiencies, respectively. *Enterobactericiae* produces H_2 at similar efficiency from different monosaccharides during growth. Among methylotrophs, methanogenes, rumen bacteria, and thermophilic archae, *Ruminococcus albus*, is promising (2.37 mol/mol glucose). Immobilized aerobic *Bacillus licheniformis* optimally produces 0.7 mol H_2/mol glucose. Photosynthetic *Rhodospirillum rubrum* produces 4, 7, and 6 mol of H_2 from acetate, succinate, and malate, respectively. Excellent productivity (6.2 mol H_2/mol glucose) by co-cultures of *Cellulomonas* with a hydrogenase uptake (*Hup*) mutant of *R. capsulata* on cellulose was found. Cyanobacteria, viz., *Anabaena*, *Synechococcus*, and *Oscillatoria* sp., have been studied for photoproduction of H_2. Immobilized *A. cylindrica* produces H_2 (20 ml/g dry wt/h) continually for 1 year. Increased H_2 productivity was found for *Hup* mutant of *A. variabilis*. *Synechococcus* sp. has a high potential for H_2 production in fermentors and outdoor cultures. Simultaneous productions of oxychemicals and H_2 by *Klebseilla* sp. and by enzymatic methods were also attempted. The fate of H_2 biotechnology is presumed to be dictated by the stock of fossil fuel and state of pollution in future.

KEY WORDS: hydrogen production, facultative anaerobe, *Clostridium* sp., *Anabaena* sp., cyanobacterium, nitrogen fixation, electron donors, hyperthermophilic archaeon, hydrogenase, mixed culture, immobilization, photosynthetic hydrogen, methanogen, methylotrophs, hydrogen and oxychemicals, outdoor culture, photosynthetic aututrophs, and heterotrophs.

I. INTRODUCTION

The production of hydrogen by bacteria has been known.[1,2,3,4] Development of process for H_2 production by microorganism did not receive attention in parallel to extensive basic studies on microbial H_2 metabolism. With the increasing burning of fossil fuel and consequent possible changes in global climate, the use of H_2 as the safe fuel is frequently suggested, as it produces only water on burning.[5,6,7] Reports on the microbial production of H_2 have been periodically reviewed by various workers[8,9] and recently Beneman[10] critically assessed the prospect of H_2 biotechnology based on the activities of photo and nonphotosynthetic bacteria. He favored photosynthetic process rather than nonphotosynthetic microbial processes, which produced H_2 less efficienctly from carbohydrate substrates. However, production of

H_2 by the dark pr ... ...pler than phot processes on technical ground and the dark processess generate H_2 from a large number of carbohydrates frequently obtained as refuse or waste products. This article reviews the status of different microbial H_2 production processes so far reported, particularly with respect to their production efficiencies and raw material utilizations.

The production of H_2 by different microorganisms is intimately linked with their respective energy metabolisms. In aerobic microorganisms, released electrons from substrate oxidation is transferred to oxygen as the ultimate oxidant, while in anaerobic microorganisms electrons released from anaerobic catabolism use many terminal oxidants such as nitrate, sulfate, or organic compounds derived from carbohydrates as the carbon source. The production of H_2 is one of the specific mechanisms to dispose excess electrons through the activity of hydrogenase present in H_2 producing microorganisms.

Gray and Gest[11] categorized all hydrogen producing microorganisms into four groups:

1. Strict anaerobic heterotrophs that do not contain a cytochrome system (*clostridia, micrococci, methanobacteria*, etc.)[12,13,14]
2. Heterotrophic facultative anaerobes that contain cytochromes and lyse formate to produce H_2[15,16]
3. *Desulfovibrio 'desulfuricans* is the only strict anaerobe in this group with a cytochrome system.[17]
4. Photosynthetic bacteria with light-dependent evolution of H_2 from reduced NADH.

They suggested that coupling of hydrogen formation was more intimate in group 1 organisms where electrons were disposed from energy-yielding oxidation. The above closeness, however, was not evident in group 2 organisms, where disposal of the electron by H_2 formation promoted energy-yielding oxidation by removing the end-product formate.

Group 3 organisms were supposed to possess both the mechanisms for H_2 production. Kosaric and Lyng[18] compiled an extensive list of heterotrophic bacteria, which were reported to produce hydrogen.

II. PRODUCTION OF HYDROGEN

A. Anaerobes

1. Clostridia

The obligate anaerobic clostridia lack a cytochrome system for oxidative phosphorylation and generate ATP by substrate level phosphorylation during fermentation. Glucose, in glycolytic pathways, generates ATP and NADH with the formation of pyruvate. Pyruvate produces acetyl CoA, CO_2, and H_2 by the activities of pyruvate-ferredoxin-oxidoreductase and hydrogenase (HD). NADH is used in the formation of butyrate from acetyl CoA with concomitant generation of ATP by phosphobutyrylase and butyrate kinase.[19] Acetyl CoA also generates ATP by acetyl kinase and NADH is also oxidized to produce H_2 by ferredoxin-oxidoreductase, ferredoxin, and HD.[20,21] The possible catabolic routes have the stoichiometry.

i) Glucose $\rightarrow 2H_2$ + butyrate + $2CO_2$

ii) Glucose + $2H_2O$

$\rightarrow 4H_2$ + acetate + $2CO_2$
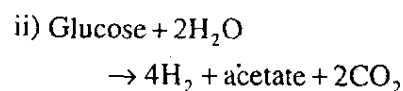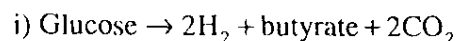
The available H_2 from glucose is determined by the ratio butyrate/acetate produced during fermentation.

In the early 1960s Magna Corporation reported fermentative production of H_2 in a 10 l fermentor using strains of *C. butyricum* and *C. welchii*.[22] Karube et al.[23] immobilized whole cells of *C. butyricum* IFO 3847 in polyacrylamide gel that produces 0.63 mol H_2/mol of

glucose in 24 h. H_2 production dropped spontaneously, however, due to the accumulation of organic acids. Brosseau and Zajic[24] reported H_2 production efficiency of 1.5 mol H_2/mol of glucose during stationary growth phase of *C. pasteurianum* in a 14 dm³ batch reactor. A higher H_2 productivity (1.8 to 2.0 mol/glucose) by the growing culture of a newly isolated *C. beijerincki* AM21B strain was reported by Taguchi et al.[25] The bacterium produced H_2 not only from glucose but also from starch at comparative rates. However, sustained production of H_2 was not achieved and H_2 production dropped before the exhaustion of carbohydrate in the medium. The strain, however, could utilize a large number of carbohydrates such as arabinose, cellobiose, fructose, galactose, lactose, sucrose, and xylose with the efficiencies from 15.7 to 19.0 mmol/g of substrate over 24 h.[26] Another *Clostridium* species[27] isolated by the group which H_2 more efficiently from xylose and arabinose (13.70 to 14.55 mmol/g) than from glucose (11.07 mmol/g). This suggests the possibility for the production of H_2 from abundant hemicellulosics of plant biomass. Taguchi et al.[28] studied the possibility of H_2 production from the enzymatic hydrolysates of Avicel and xylan, by the *Clostridium* strain. H_2 was produced at the rates of 16.1, 14.6, 19.6, and 18.6 mmol/g of pure xylose, glucose, and enzymatic hydrolysate of Avicel and xylan, respectively. However, the simultaneous presence of crude xylanase and xylan in the medium gave a lower production rate of 9.6 mmol of xylose. The production of H_2 was also studied[29] in a two-phase continuous system with cellulose hydrolysate. The system was studied for 100 h using two phases of 10% polyethylene glycol- 50,000 and 5% dextran- 40,000. H_2 production rate with Avicel hydrolysate was 4.10 mmol/h compared with 1.78 for glucose. The stoichiometry of H_2 yield (mol/mol glucose) was as high as 4.46 for avicel hydrolysate compared to 2.14 for glucose. To minimize the cost of cellulase preparation in H_2 production, attempts to isolate novel cellulolytic H_2-producing bacteria was suggested. However, in continuous fermentation of 3% (w/v) xylose and glucose, optimum H_2 production rates were improved to 21.03 and 20.40 mmol/h/l, respectively, and 2.6 mol and 2.36 mol of H_2 per mol of glucose and xylose were formed.[30] Taguchi et al. also isolated another *Clostidium* sp. strain X53, which produced both xylanase and H_2 in xylan medium. The optimum xylanase production was 1252U/ml after 8 h cultivation at 40°C and the maximum H_2 evolution rate was 240 ml/l/h. However, total yield of H_2 was 23% less than that obtained from equivalent amount of xylose present in xylan.[31]

The production of biochemical fuel cell by the use of H_2 producing *Clostridium butyricum* from glucose was attempted by Rohrback et al.[32] Suzuki et al.[33] immobilized cells of *C. butyricum* in 2% (w/v) agar gel and studied H_2 production from wastewater from an alcohol factory. The operation was continued for 20 d and a current of 15 mA was obtained. Later the group[34] improved their process by using a system consisting of a continuously stirred reactor containing immobilized cells of *C. butyricum*, fitted with two gas type hydrogen-air (oxygen) fuel cells. Wastewater from that alcohol factory, which used molasses as the raw material, was applied to the system. The study was done with 1 kg of immobilized whole cells in a fermentor of 5 l capacity. The optimum H_2 production rate was 7 ml/min with wastewater of BOD value as 1500 ppm. Although the rate was further increased up to 10 ml/min with a higher stirring rate of the reactor the higher agitation was associated with significant crushing of the gels. The observed fall of H_2 production with the lowering of BOD of the medium with time was avoided by the continuous addition of condensed wastewater. Production rate was also decreased with the lowering of pH with time. In operation, total replacement of wastewater was done after a 2 h interval over 20 d. About 63% of

total sugar (gluco ... ...)as pr sent in waste-
... was con erted into H with th ideal
production rate as 2 mol H₂/m... ' , glucose.

2. Methylotrophs

In 1979, Egorov et al.[35] first isolated NAD-
dependent formate dehydrogenase (FDH) from
a methylotrophic bacterium and indicated the
possibility for the development of the system
for NADH regeneration or H_2 production from
organic fuel. Later, Kawamura et al.[36] stud-
ied production of H_2 by CH_4 utilizing bacteria
Methylomonas albus BG8 and *Methylosinus*
trichosporium OB3b under anaerobic condi-
tions. They examined production of H_2 from
various organic substrates viz., methane, meth-
anol, formaldehyde, formate, pyruvate, etc. Of
these substrates, formate was the most suitable
substrate for the production of H_2 under anaero-
bic conditions. *M. albus* and *M. trichosporium*
produced 2.45 and 0.61 μmol H_2/μmol of for-
mate after 5 h incubation. The H_2 producing
system was suggested to involve NAD-depen-
dent FDH and HD, which are constitutive and
soluble enzymes for the strain. They also stud-
ied H_2 production by another methanol-utiliz-
ing bacterium *Pseudomonas* AMI but no H_2
was evolved similar to another strain, *Pseudom-*
onas methylica, reported earlier.[37]

3. Methanogenic Bacteria

Although the presence of hydrogenase is
characteristic of this group of organisms, meth-
anogens usually oxidize H_2 as a sole source
of energy for CH_4 production and for reduc-
tive assimilation of CO_2 into cellular carbon.
Zehnder et al.[38] isolated a methanogen that
was capable of lysing formate. The strain ini-
tially characterized as *Methanobacterium so-*
ehngenii could grow in mineral salt medium
with acetate as the organic substrate. CH_4 was
exclusively generated from a methyl group
of acetate and organisms capable of splitting

formate and NADP-linked HD activities were
also found in the cell extract. Huser et al.[39]
later characterized the strain as *Methanotrix*
soehngenii. The strain could not utilize for-
mate as a carbon source but was capable of
splitting formate into equimolar proportions of
H_2 and CO_2. No further investigation, how-
ever, was reported on the production of H_2 by
the strain. Bott et al. reported production of
H_2 and CO_2 in stoichiometric amounts of CO
and H_2O by a strain of *Methanosarcina bark-*
eri in presence of bromoethane-sulfonate, which
specifically inhibited CH_4 formation.[40]

4. Rumen Bacteria

Ruminococcus albus, an anaerobic rumen
bacterium capable of hydrolyzing cellulose,
was known to produce acetate, ethanol, for-
mate, H_2, and CO_2 from carbohydrate.[41-43]
Miller and Wolin[44] estimated fermentation prod-
ucts from glucose by the cells of *R. albus*. H_2
was produced at the rate of 59 mmol/100 mol
glucose along with the acetate, ethanol, and
formate that accumulated in the medium. Pyru-
vate was converted into H_2 (~0.8 mol/mol)
by washed cells, but formate was neither ob-
tained from pyruvate nor lysed by the cells to
H_2 and CO_2. They suggested that the H_2 pro-
ducing pyruvate lyase, as present in *E. coli*,
was functional in *R. albus*. Innotti et al.[45] re-
ported production of H_2 from glucose by
R. albus in continuous culture. Products ob-
tained per 100 mol of glucose during growth
of *R. albus* were 65 mol of ethanol, 74 mol of
acetate, and 237 mol of H_2. However, *R. albus*
was not studied further for H_2 production.

5. Archaea

Microbial HD linked with the oxidation or
evolution of molecular H_2 are all iron-sulfur
protein, and those associated with membrane-
bound electron transport system contain nickel
and usually function to oxidize hydrogen.[46,47]

HD without nickel is a soluble enzyme and linked with low potential cytochromes or ferredoxin.[48] The hyperthermophilic archeon *Pyrococcus furiosus*, however, contains soluble nickel containing HD and produces H_2 from carbohydrate and peptide.[49] The strain was reported to oxidize pyruvate,[50] aldehyde,[51] indolepyruvate,[52] formaldehyde,[53] and 2-ketoglutarate.[54] Ferredoxin linked oxido-reductases were suggested to be involved in the oxidation and reduced ferredoxin was recycled with generation of H_2 or H_2S either via HD or sulfhydrogenase.[55] Ma et al.[56] studied production of H_2 from pyruvate by the enzymes purified from *P. furiosus*. They showed that production of H_2 from pyruvate involved participation of pyruvate-ferredoxin oxido-reductase, followed by transfer of electron from reduced ferredoxin to NADP. The enzyme ferredoxin: NADP oxido-reductase (sulfide dehydrogenase) also reduced elemental sulfur by NADPH as an electron donor. The production of H_2 from NADPH was catalyzed by HD, which was also a sulfur reductase or sulfhydrogenase. The H_2 evolving system of *P. furiosus* appeared to be unusual compared with those present in other bacterial systems. The organism was reported to grow optimally at 100°C and produce organic acid, CO_2, and H_2 from carbohydrate or peptide, but H_2 production efficiency of the organism was not evaluated.

B. Facultative Anaerobes

1. Escherichia coli

The anaerobic decomposition of formate into H_2 and CO_2 by *E. coli* was studied extensively by Stickland[57-60] and Yudkin[61] during 1929 to 1933. The catalytic activity termed as 'formate hydrogenlyase' (FHL) was found to be inducible in *E. coli* and washed cell suspension decomposed formate anaerobically into equimolcular amounts of H_2 and CO_2.[57] Presence of O_2 or methylene blue, however, caused decomposition of formate without liberation of H_2. Aeration had an inhibitory effect on

induction but not on the catalytic activity of FHL system.[59] Later, the FHL system was shown to be a membrane-bound multienzyme system consisting of a FDH and a HD linked by unidentified electron carriers.[11,61-63] The FDH linked to H_2 production was active on one electron dye benzyl viologen (BV) unlike other FDH capable of reducing methylene blue (MB).[64] FDH (BV) catalyzed the non-energy yielding reaction was repressed by O_2, NO_3^-, and MB.[65,66] Formate could be oxidized without H_2 production by FDH (MB) and linked to different anaerobic reductase systems ($NO_3^- \rightarrow NO_2^-$ and fumarate $\rightarrow$ succinate) with the generation of energy as ATP.[67,68,69]

Klibanov et al.[70] advocated the use of FHL system in the reversible reaction catalyzed by bacterial FHL system ($HCOO^- + H_2O \Leftrightarrow H_2$ $H_2 + HCO_3^-$) for formation of H_2 from formate as well as for transportation of H_2 as formate. The immobilization of FHL system of *E. coli* and sustained stoichiometric conversion of formate into H_2 and CO_2 were reported by Nandi et al.[71] They showed production of H_2 from 1.15 M formate over a 96 h cycle with loss of 25% efficiency per cycle. The system required presence of a small amount of glucose, which was converted into succinic acid. The immobilized cell also synthesized formate (224 mg/g wet cell) from H_2 and CO_2 mixture. In earlier studies, Peck and Gest[62] indicated that activity of FHL as present in cell-free lysate of *E. coli* needed activation of FHL system by the addition of carbohydrate or C_2 compounds of carbohydrate metabolism. Nandi et al.[72] showed that sustained lysis of formate required blocking of other anaerobic reductases present in *E. coli*. They proposed that the redox potentials of the electron transport carrier as present in FDH to HD and those present in the anaerobic reductase systems (Fumarate $\rightarrow$ Succinate, Tetrathionate $\rightarrow$ Thiosulfate) might be overlapping, causing leakage of electron flow from FHL system to the reductase. Presence of succinate or thiosulfate as terminal reduced product also

prevented the lea · ..:.l ·u;· ·ed stoichi-
oi·cti·ic ·., ·'·::::i·icd lysis of forn.·'·.

The production of H_2 from c · `· .·rate by
washed *E. coli* cell was reported i·y Stickland.[52]
Anaerobic decomposition of glucose, fructose,
and mannose was similar to that of formate
while lactose, galactose, arabinose, glycerol,
and mannitol generated H_2 at lower yield. They
indicated, however, that the production of H_2
from glucose had not occurred through for-
mate as the intermediate. However, Ordal and
Halvorson[73] compared H_2 production from
sugars and formic acid by normal and variant
strains of *E. coli* and showed that H_2 from
glucose definitely came from formic acid, which
is an intermediate in the generation of H_2 by
the bacteria. Blackwood et al.[74] also reported
0.72 to 0.91 mol/mol conversion of glucose
into H_2 by various pigmented and nonpigment-
ed strains of *E. coli*. The anaerobic production
of H_2 from carbohydrate by growing cells of
E. coli through formate always have low con-
version rates as formate is not the sole end
product from glucose. Observation on carbon
balance showed production of 90 mmol of
ethanol and acetate, 90 mmol of H_2 and for-
mate, and 15 mmol of CO_2 and succinate from
100 mmol of glucose by growing *E. coli*.[74,75]
It was found[68,69] that pyruvate generated from
glucose metabolized in two stages in absence of
any electron acceptor-like nitrate or fumarate.
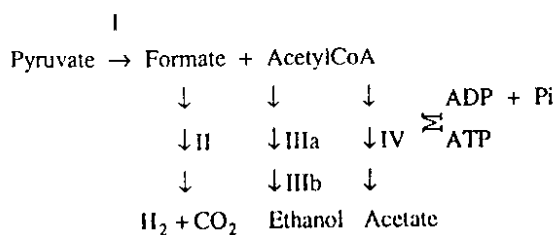
I   = Pyruvate formate lyase
II   = FDH(BV) → X_1 — X_2
       → Hydrogenase(FHL)
IIIa = Aldehyde : NAD Oxido-reductase
IIIb = Alcohol : NAD Oxido-reductase
IV = ATP   : Acetate phototransferase

It was found possible to achieve 1.2 stoichi-
ometry from glucose by using immobilized
whole cells of *E. coli* containing FHL activity
(Nandi et al., unpublished).[76]

2. Enterobacter

Tanisho et al.[77] isolated a strain of *Enter-
obacter aerogenes* that produced H_2 at 38 to
40°C in a medium containing glucose, pep-
tone, and salts. The highest productivity achieved
was 0.20 to 0.21 l H_2/h/l of medium. Later the
strain was designated as *E. aerogenes* and pro-
duction of H_2 was optimized to 0.52 l H_2/h/l
of medium over a period of 23 h. The stoichi-
ometry of H_2 production was mol/mol of glu-
cose.[78] The influence of pH and biomass pro-
ductivity with H_2 generation by the strain were
also studied. With glucose as the carbon source,
the highest H_2 evolution rate was 13 mmol H_2/g
dry wt cell/h at 38°C.[79,80] A strain of *Entero-
bacter aerogenes* capable of growing at acidic
pH (3.3 to 4.0) was reported by Yokoi et al.[81]
The strain utilized glucose, galactose, fructose,
and mannose for H_2 production at mol/mol con-
version rate. The strain also used dextrin for H_2
production at the similar conversion rate. The
production was also studied in continuous cul-
ture over a 26-d period. Evolution of H_2 took
place at the average rate of 120 ml/h/l medium
with the conversion rate of 0.8 mols H_2/mol
of glucose. The fall of H_2 production observed
in the later phase was supposedly due to the
inhibitory activity of accumulated acids such
as acetic, succinic, and lactic acids.

C. AEROBES

1. Alcaligenes

The aerobic H_2 bacteria have the ability to
utilize H_2 and CO_2 as the sole source of ener-
gy and carbon, respectively. The organisms
contain a soluble NAD-reducing HD and can

grow heterotrophically.[82-84] Kuhn et al.[85] showed that *Alcaligenes eutrophus* grown heterotrophically on gluconate or fructose, when exposed to anaerobic condition, evolved molecular H_2 from organic substrate. *A. eutrophus* contains soluble NAD-reducing HD, which reduces NAD directly with H_2 and disposes excess reductant in the form of H_2 when grown under anaerobic condition. Electrons derived from the catabolism of organic substrate do not enter into the respiratory chain.[86] Klibanov et al.[70] immobilized cells of *A. eutrophus* in kappa-carrageenan and studied reversible reaction.

$$HCOOH \Leftrightarrow H_2 + CO_2$$

In the decomposition of formate, higher concentration of formate (>0.5 M) inhibited H_2 production. Although the immobilized cells had good storage stability, the sustained lysis of formate by the immobilized cells was not demonstrated.

2. Bacillus

A hydrogen-producing culture of *Bacillus licheniformis* was isolated by Kalia et al.[87] from a mixed culture of H_2-producing bacteria from cattle dung.[88] In batch culture, *B. licheniformis* produces 13 l H_2/mol of glucose in 24 h from 3% (w/v) glucose in the medium.[89] The cells were immobilized on brick dust and in calcium alginate beads. Alginate beads had the H_2 production efficiencies as 16 l/mol glucose/d compared with 31 l/mol/d with cells immobilized on brick dust. The immobilized cells were stable over 60 d in a continuous system and an average conversion ratio of 1.5 mol H_2/mol glucose was achieved.

D. Photosyntheticc Bacteria

Photosynthetic bacteria are capable of reducing CO_2 by the reductants, derived from various organic and inorganic sources.[90] Among the photosynthetic bacteria, the purple sulfur bacteria (*Thiocapsa* and *Chromatinum*) are obligate anaerobic autotrophs, which utilize H_2, H_2S, and elemental sulfur, whereas non-sulfur *Rhodospirillum* and *Rhodopseudomonas* could not use sulfur and are capable of growing aerobically on organic substrate in absence of light. The production of H_2 by *Rhodospirillum rubrum* during photosynthetic growth on various compounds was studied extensively by Gest and his workers.[91-94] In a medium containing limiting amounts of ammonium salt, H_2 production began after the exhaustion of ammonium salt and production was associated with photometabolization of organic substances in absence of significant growth. Efficient H_2 production, however,occurred in the presence of glutamate as the nitrogen source.[91] Washed cells grown in the presence of glutamate evolved H_2 from Krebs cycle acid under photo illumination.[92,93] Resting cells of *R. rubrum*[94] released H_2 more or less quantitatively from acetate (4 mol), succinate (7 mol), fumarate (6 mol), and malate (6 mol). Stoichiometric release of H_2 possibly[95] occurred due to the presence of highly active anaerobic citric acid cycle coupled with photoreaction that efficiently oxidized reduced NAD^+ generated by the citric acid cycle. All photosynthetic bacteria, however, use H_2 as a reductant for the fixation of CO_2 and are capable of fixing molecular nitrogen. It was subsequently understood that nitrogenase had the dual activities for nitrogen reduction as well as for ATP-dependent H_2 evolution.[96-100] It was suggested that H_2 production took place when cells produce excess ATP and reducing capability of the cells surplused the demand, due to the presence of readily available carbon sources (Krebs Cycle acid) or reduced nitrogen source like glutamate/aspartate.[101] HD present in photosynthetic bacteria, which is involved in the utilization of H_2 as a source of reductant for CO_2 fixation, is distinctly different

from nitrogenase.[102-106] The functional relationship between HD and nitrogenase is complicated. It was proposed that enzymes were genetically linked in *Rhodopseudomonas acidophilla*.[107] HD of *Rhodopseudomonas capsulata*, however, was found to be constitutive.[100] Several species of *Rhodospirillaceae* that were capable of growing in the dark on glucose, organic acids, including formate with the production of H_2 and CO_2 also indicated non-nitrogenase-mediated production of H_2. Later, it was shown that non-sulfur bacteria in dark growth had pyruvate: formatelyase and FHL activities similar to those of *E. coli*.[106-109] It is not known whether HD isoenzymes involved in the oxidation or production of H_2 as detected in *E. coli* were present.[108-113] The H_2 uptake membrane-bound HD of *Rhodobacter capsulata*,[114,115] *Thiocapsa roseopersicina*,[116] and *Rhodospirillum rubrum*[117] were all Ni-Fe-HD similar to that of H_2-uptake HD of *E. coli*.[118] *R. rubram* was known to contain several HD induced by CO,[119] CO_2/H_2,[120] pyruvate[121] and also the HD, which was induced under nitrogen fixing condition.[117] HD induced by CO was characterized[122] to be a Ni-Fe HD with closest similarity to *E. coli* isoenzyme 3.[123] Gest and Kamen[92] reported that *R. rubrum* grown photosynthetically with glutamate or aspartate instead of ammonium ion produced H_2 from malate, fumarate, or oxaloacetate at mol to mol ratio. Although formate was not lysed by the cells, organisms adapted in formate could produce H_2 and CO_2 in the dark. Since the report of Gest and Kamen,[92] nonsulfur purple bacteria were not recognized for the production of H_2 over 25 years. Hillmer and Gest[124] initiated studies on *Rhodopseudomonas capsulata* for H_2 production in presence of glutamate. Various amino acids, except lysine and cystein as nitrogen source, supported H_2 production and optimum H_2 production rate achieved was 130 µl/h/ml of culture. Later, they showed that resting cells could produce H_2 from C_4 acid, lactate, pyruvate but not from C_3 acids. They

suggested that production of H_2 and that for reduction of CO_2 were catalyzed by different enzyme systems.[125] Weetall et al.[126] immobilized *R. rubrum*, which was contaminated with *Klebseilla pneumoniae*. The immobilized cells in agar gel produced H_2 from glucose and cellulose hydrolysate. The system was continuously studied in a reactor for 30 d and the half-life period was estimated to be 1000 h. The efficiency of the system varied from 21 to 89% on the basis of a theoretical production rate at 6 mol H_2 per mol glucose. Watanabe et al.[127] isolated different strains of *Rhodopseudomonas gelatinus* and *Rhodopseudomonas sphaerolides* and studied their efficiencies for the production of H, from glutamate-malate medium. Highest H_2 production efficiency was reported to be a 90 µl/h/mg cell.

Kelly et al.[128] studied the production of H_2 by *Rhodopseudomonas capsulata* and showed that H_2 produced by nitrogenase was directly recycled by HD under low substrate concentration. Zurrer and Bachofen[129] reported continuous production of H, by *R. rubrum* from lactate, whey, or yogurt waste up to an 80 d period under illumination with periodic addition of lactate. The average production rate was 6 ml/h/g (dry) cell with efficiencies of 67 to 99%, depending on the substrate used. The production rate was improved to 20 ml/h/g cells in continuous culture with 70 to 75% efficiency of H_2 production.

Macler et al.[130] reported the isolation of *Rhodopseudomonas sphaeroides* mutants that were capable of converting glucose quantitatively into H_2 and CO_2. The mutant, unlike the wild strain, did not accumulate any gluconate from glucose. The production was studied over a period of 60 h with optimum production rate obtained between 20 to 30 h of growth.

Kim et al.[131] isolated a few *Rhodopseudomonas* sp. that produced higher amounts of H_2 (130 ml/h/mg cell) from a gluconate-malate medium. Odom and Wall[132] reported production of H, from cellulose using co-cultures of *Cellulomonas* sp. strain ATCC

21399 and *Rhodopseudomonas capsulata*. They used both wild and an uptake HD (*Hup⁻*) lacking mutant of the phototroph. The gas production was studied over a 200 h period under anaerobic and illuminated conditions. The co-culture of *Cellulomonas* with *Hup* mutant produced 4.6 to 6.2 mol H₂/ glucose compared with 1.2 to 4.3 mol produced by wild phototroph under same conditions.

Later, Segers et al.[133] studied production of H₂ and CO₂ from lactate, acetate, and butyrate by axenic cultures of *Rhodopseudomonas capsulata, Rhodospirillum rubrum*, and *Rhodomicrobium vannielii* with glutamate or dinitrogen as nitrogen sources. Cells were grown in a medium containing 30 mM organic acid and 7 mM glutamate under illuminated condition. The theoretical conversion yields were supposed to be the amounts of available H₂ present in lactate, acetate, and butyrate. The productivities ranged from 100 to 926 ml H₂/l/d (l = volume of medium) with conversion efficiencies from 23 to 100%. Replacement of glutamate by N₂ gas improved productivity up to 760 ml/l/d and 100% efficiencies were achieved in all cases. The fermentation was continued for 10 d. On aging, nitrogenase activity decreased slowly with an increase of H₂ oxidizing activity of the strains, particularly when gaseous N₂ was used in place of glutamate.

Willison et al.[134,135] reported nitrogenase mediated H₂ production efficiencies of a few mutants of *Rhodopseudomonas capsulata* B10 isolated by chemical mutagenesis of wild strain. Three mutants showed increased H₂ production over the theoretical stoichiometry. The mutant IR4 produced 10 to 20% more H₂ with DL-lactate or L-malate, 20 to 50 % more with DL-malate and up to 70% more with D-malate, compared with the wild strain. The strain was found to be deficient in membrane-bound HD activity as measured by H₂-dependent reduction of MB or BV. It was suggested that overproduction of H₂ and CO₂ by the mutant

was possibly due to altered carbon metabolism. It was also found that the activity of NAD⁺-dependent malate dehydrogenase was 50% more in mutants, which grew at much faster rate than the wild strain in a medium containing D-malate. Hirayama et al.[136] immobilized whole cells of *R. rubrum* G-9 BM in carrageenan or agar gel that were reported to be highly stable over a long time. The production of H₂ from a large number of substrates viz., different organic acids, sugar, and sugar alcohol was studied in a designed continuous reactor with intermittent feeding over a 60 d period. Highest production (13.74 ml/ 48 h/20 mg cell) was achieved with butyrate and lowest of 2.68 ml with sorbitol. The rate of H₂ production initially observed, however, dropped to 40% of the highest within a few hours, but the resultant rate was more or less steady for the subsequent period, indicating the problem for the maintenance of immobilized bead structure and pH over a long period of H₂ production.

A strain of *Ectothiorhodopsira vacuolata*, a purple phototroph capable of oxidizing reduced inorganic sulfur compounds and elemental sulfur, was isolated by Chadwick and Irgens.[137] In the media containing limiting NH₄Cl, hydrogen was produced from acetate, pyruvate, propionate, fumarate, malate, and succinate under illumination. Optimum H₂ production rate was 16 ml of H₂/25 ml of the culture. The concentration of sodium sulfide and intensity of light were reported to have significant effects on H₂ production. Wright et al. (1991) reported H₂ production during photocatabolism of aromatic compounds (benzyl alcohol, vanillate, and syringate) by *Rhodomicrobium vanniellii*.[138] Later Fibler et al.[139] studied H₂ production from different aromatic acids by *Rhodopseudomonas palustris*. Under limited concentration of glutamate (1 mM), the strain produced H₂ from benzoate, *p*-hydroxybenzoate, cinnamate, and mandelate. Production of H₂ was also increased with increased nitrogenase activity, but not by inhibi-

tion of *hup⁻* HD by ΓINTA I . . . te or man-
delate yielded 32 to 45% of theoretical amounts
of H_2 by the different strain. *. . .stris*
DSM 131 also immobilized in agar, agarose,
κ-carrageenan, and sodium alginate gels. With
alginate the yields of H_2 production were 60%,
57%, 86%, and 88% of the theoretical amounts
from mandelate, benzoylformate, cinnamate,
and benzoate.[140] H_2 production by *T. rubrum*
was found to be stimulated threefold by the
inactivation of *Hup⁻* activity and the addition
of 0.5 mM EDTA to the medium. Fe^{2+} and
Fe^{3+} also increased H_2 production by stimu-
lating nitrogenase activity of *T. rubrum*.[141]

It was suggested that biosynthesis of poly-
hydroxybutyric acid, an intracellular storage
compound, and photoproduction of H_2 possi-
bly compete for reducing equivalent in *Rho-
dobacter sphaeroides*.[142] In PHB-negative mu-
tant, the effect was insignificant with lactate
but pronounced with acetate as the substrates
for H_2 production.

Production of H_2 in a nozzle-loop bioreac-
tor was studied by Seon et al.[143] with *R. ru-
brum* KS-301 immobilized in calcium algi-
nate. In the continuous glass-reactor (2 l) glucose
concentration was varied from 0.5 to 5.4 g/l
over a period of 70 h at 30°C. The optimum H_2
production rate achieved was 91 ml/h at the
dilution rate of 0.4 ml/h for 70 h with initial
glucose concentration of 5.4 g/l.

The kinetics of substrate utilization by im-
mobilized cells of *Rhodopseudomonas capsu-
lata* 366 and *Rhodopseudomonas* sp. D was
studied by Xu et al.[144] H_2 production efficien-
cy was better in agar gel than in alginate. The
production of H_2 did not proceed simultane-
ously with substrate utilization, but was gov-
erned by biochemical reactions. In an immo-
bilized bio-reactor fed with glucose and lactate,
H_2 production was 0.659 l and 0.477 l/d, respec-
tively, with strains 386 and D. The gas produc-
tion increased up to 1 l/d when lactate was used
alone.

Jahn et al. (1994) showed that *HupL* mu-
tant of *Rhodobacter capsulatus* B10 cannot

grow photoautotrophically and evolve H_2 by
the activity of nitrogenase under photohetero-
trophic growth condition in limited nitrogen
containing medium. The mutant liberated H_2
from DL-malate, D-malate, and L-lactate by more
than 90% of the theoretical yields compared
with 54 to 64% by the wild B10 strain.[145] Re-
cently, the role of alternative nitrogenases[146]
in *Rhodobacter capsulatus* on H_2 production
were investigated. It was shown that *R. cap-
sulatus* contained normal Mo containing nitro-
genase as well as Fe containing nitrogenase,
which was expressed under extreme Mo de-
ficiency.[147] Krahn et al.[148] compared H_2 pro-
duction by the Mo-nitrogenase and alternative
Fe-nitrogenase of *R. capsulatus hup⁻* mutants.
They reported a comperative study with cell
suspension of *hup⁻* mutant and *nif*HDK (lack-
ing the genes for encoding Mo-nitrogenase
protein) deletion mutant. It was shown that
hup⁻ mutation did not affect nitrogenase ac-
tivity but increased H_2 production in the wild
strain significantly and more prominantly in
Δ *nif* HDK mutant.

E. Cyanobacteria

Cyanobacteria (blue-green algae), the oxy-
genic phototrophic bacteria, perform photo-
synthesis through photosystems I and II much
like higher plants.[149] Most of the cyanobacte-
ria possess a nitrogenase system for H_2 pro-
duction. The expression of nitrogenase sys-
tem, however, required special growth conditions
and deficiency of a combined nitrogen source.
Some cyanobacteria have the capacity to form
heterocysts that lack a water-splitting photo-
system and produce H_2 through the nitrogenase
system under limiting N_2 concentration.[150,151]
Nonheterocystous cyanobacteria, however, re-
quire both N_2 deficiency and anoxic condition
for H_2 production. Although H_2 production
by cyanobacteria is supposedly due to nitrogen-
ase,[152] hydrogenases involved in H_2 produc-
tion were identified in *Oscillatoria limneti-
ca*[153] and *Anabaena cylindrica*.[154] In the

population of cyanobacteria, a hetrocystous percentage could be increased in the presence of various chemicals such as 7-azatryptophan,[155] 3α-amino-1,2,4-tryazole and N'-(3,4-dichlorophenyl)-N-α-dimethyl urea[156] that inhibited the water splitting photosystem. Liberations of H_2 and O_2 by the photosystem are also decreased in the presence of CO_2, C_2H_2, and Ar in the gas phase. The production of H_2 by the photosystems of nonnitrogen-fixing cyanobacteria is low compared with those containing a nitrogenase system. In heterocystous cyanobacteria such as *Anabaena* and *Nostoc*, the nitrogenase system was not impaired by the evolution of O_2 by the vegetative cells and organisms could produce H_2 in the presence of light.[18] Nonheterocystous filaments of cyanobacteria produce H_2 on alternative exposure to light and dark. In light, bacteria fix CO_2 into storage polysaccharide and evolve O_2. Under axonic dark conditions, nitrogenase is formed and storage polysaccharide is catabolized to provide electrons for nitrogen fixation and H_2 evolution.[157] Although large numbers of cyanobacteria were reported to produce H_2,[18] *Anabaena cylindrica* and *Synechococcus* sp. were studied extensively for H_2 production.

In 1974, Benemann[158] reported that actively growing cells *A. cylindraca* could photolyze H_2O into H_2 and O_2 and the process was strongly inhibited by N_2, and slightly by CO and CO_2. H_2 evolution under Ar was highest and linear up to 3 h. The nitrogen starved cells of *A. cylindrica* produced H_2 and O_2 up to 19 d with optimum production of 32 μl/h/mg dry wt of cells. The addition of NH_4^+ (10^{-4} to 5 × 10^{-5} M) increased total H_2 production, but decreased the H_2/O_2 ratio from 4:1 to 1.7:1.[159] Later, the influence of CO (3%), CO_2 (2%) and C_2H_2 (10%) v/v in presence of Ar or air on H_2 production revealed that the Ar-CO_2 combination had the highest activity followed by air, CO, CO_2,C_2H_2 combinations. Higher cell density increased H_2 production up to 8 μmol/h/40 mg of dry wt of cells.[160] Smith

and Lambert[161] also performed an outdoor culture of *A. cylindrica* B629 on small glass bead under a gas phase of CO_2 (0.2%), C_2H_2 (5%), O_2 (6.5%) in N_2 (158 l) with a medium containing 10 mM $NaHCO_3$. Total production of H_2 over 21 d of duration reached up to 1100 ml. They also carried out an extensive study on the effects of NH_4^+, O_2, CO_2, and C_2H_2 on anaerobic and aerobic H_2 formation by *A. cylindrica* B629.[162] The authors reported H_2 production rate upto 200 nmol/mg/h in air when concentration of C_2H_2 was varied in presence of 0.2% CO or concentrations of CO were changed in the presence of 10% C_2H_2. The rate was comparable to the rate observed in Ar atmosphere. In the system NH_4^+ up to 0.5 mM slightly stimulated H_2 production in contrast to inhibition observed under similar conditions in the presence of Ar.[163] Higher longevity (16 to 26 d) and H_2 production rate (100 μ mol/mg) were obtained when cells were incubated beneath Ar or N_2 supplemented with CO and C_2H_2.[164]

Aerobic H_2 production by heterocysts of *Anabaena* sp. CA and IF was reported by Xiankong et al.[165] In the gas phase containing 1% CO_2 in air, optimum production of H_2 was 19 and 260 μl/mg dry wt/h by CA and IF. The strains also differed with respect to sensitivity toward the action of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea and light intensity on H_2 production. Benemann et al.[166] showed a 1.2% solar energy conversion rate by *A. cylindrica* during continuous culture for 18 d at the evolution rate of 40 μl of H_2/h/mg dry wt of cells. Kumazawa and Mitsui,[167] however, reported that *Oscillatoria* sp. Miami BG7 was superior to *A. cylindrica* B629 in the production of H_2 with N_2 as the sole nitrogen source. This was attributed due to a high rate of O_2-dependent H_2 consumption activity in the heterocystous *Anabaena* than that present in nonheterocystous *Oscillatoria*, which had lower O_2 evolution and higher respiration rate. The involvement of hydrogenase in the evolution of H_2 by photolysis in *A. cylin-*

drica PCC 712? ...aczko.[168] Cells grown in high light after a 2 h anaerobic incubation evolved H₂ via reve... le HD, while this activity was absent in low-light grown cell. *In vitro*, H₂ evolution by high and low light grown cells did not differ significantly. It was suggested that reversible HD received reducing equivalents from photolysis of water and photosystem I and II participates in the H₂ production.

Asada and Kawamura[169] studied H₂ accumulation by a strain of *Anabaena* N-7363. In a stirred incubation vessel (14 cm × 6 cm), the H₂ production rate increased from 0.371 (1st day) to reach optimum of 0.765 (2nd day), followed by gradual decrease to 0.216 μl H₂/h/mg dry wt cells on 11th day. The strain was grown in combined N₂ free medium under air atmosphere supplemented with 5% (v/v) CO₂. Recently, H₂ production by a strain of *A. variabilis*, reported earlier by Kenetemich et al.[170] was studied. The photosynthetic evolution of H₂ by the strain, studied over several weeks, increased up to 148 nmol/h/mg dry wt of cells by the addition of 77mM of Tween 85. The effect was specific for Tween 85 and not observed for Tween 20, 60, and 80; however, the role of Tween 85 on photosystems or on H₂ production was not clear.[171] The photoproduction of H₂ and O₂ in closed vessels by a marine cyanobacterium *Anabaena* sp. TU 37-1[172] under high cell density conditions was studied by Kumazawa and Asakawa.[173] The photosynthetic conversion efficiency at the density of 300 μg chlorophyll/vessel of 20 μl gas phase was 2.4 to 2.2% during 12 to 24 h incubation and the volume of H₂ accumulated was 8.4 ml/vessel under atmospheric pressure after 48 h. Gas production was prolonged by the intermittent replacement of gas phase. Markov et al.[174] reported continuous H₂ production by immobilized *A. variabilis* in hollow-fiber photobioreactor under partial vacuum for a few months. Immobilization of cells was found to be better on cellulosic hydrophilic cuprammonium rayon hollow fiber than on hydrophobic polysulfone hol-

low fibers. In the laboratory bioreactor, under 270 to 300 mmHg pressure increasing CO₂ in the gas phase was found to decrease H₂ production and increased H₂ uptake. A two phase system consisting of CO₂ uptake and H₂ evolution was suggested to be feasible. CO₂ uptake at 150 to 170 ml/g dry wt/ h caused evolution of H₂ at the rate of 20 ml/ g dry wt/h. The photobioreactor was run for 1 year continuously.[175] Sveshnikov et al.[176] showed that mutants of *A. variabilis* ATCC 29413, deficient in uptake and reversible HD, were better H₂ producers than the parent strain. In a gas phase containing 25% N₂, 2% CO₂, and 75% air, mutant PK84 produced 6.91 mmol H₂/μg protein/h, which was 4.3 times higher than that by the wild strain. N₂ and CO₂ deficiencies in the gas phase improved H₂ production both by the mutant and wild strain, indicating involvement of HD in the evolution of H₂.

During the period 1977 to 1988, School of Marine Atmospheric Science, University of Miami, Florida, made an extensive effort to study production of H₂ by marine photosynthetic microorganisms.[177-179] An attempt at the development of outdoor hydrogen production was also reported. A large number of strains with high growth rates, higher biomass yield, and long term H₂ production capability were isolated. In general, nonheterocystous filamentous strains and unicellular, aerobic nitrogen-fixing strains of Cyanobacteria were found to produce higher amounts of H₂ at faster rate than the heterocystous filamentous strain.[180] *Oscillatoria* sp. Miami BG7, a nonheterocystous filamentous strain, produced H₂ at a very high rate for long time with sea water as the H₂ donor.[177,178,181] The optimum rate observed was 0.54 μmol H₂/mg/ dry wt/h. In the two-step H₂ production, cells in culture containing combined N₂ accumulated glycogen (~65% dry wt)[181] in the first step. In the next step, cells illuminated under Ar or anaerobic condition hydrolyzed glycogen to glucose, which subsequently produced H₂. Production of 9.8 mol of H₂ from 1 mol

of glucose was observed experimentally. In a semicontinuous bench scale outdoor culture vessel (10 l) containing sea water with nutrient medium yielded daily 180 mg dry wt/l/d in the temperature range from 26 to 32°C. The transfer of cells into sunlight illuminated bioreactor (5 l) with Ar in the gas phase caused sustained H_2 production.[181] Production of H_2 by the strain was saturated at low light intensities and no photoinhibition was observed in high light.[182]

Strains of *Synechococcus* were also isolated by the laboratory[183] and strain Miami BG 043511 was found to be very promising. *Synechococcus*, being a unicellular aerobic nitrogen-fixing cyanobacteria, produced H_2 and O_2 simultaneously from sea water in a single step. The strain produced H_2 maximally at 1.6 μ mol/mg dry wt/h simultaneously with O_2 at a 2:1 stoichiometry. The nonheterocystous strain did not release CO_2 and electrons released from water efficiently reduced H^+ or via rapid refixation of CO_2 released during breakdown of internal electron donor compounds.[180]

The mechanism by which nonheterocystous cyanobacteria performs both O_2 evolving photosynthesis and O_2-labile N_2-fixation in the same cell type remained unsolved for a long time. Mitsui et al.[184] showed that *Synechococcus* sp. under synchronized condition performed N_2-fixation and photosynthesis at different phases in its cell division cycle. In synchronous growth, nitrogenase activity at the onset of the incubation period did not affect H_2 production rate, but cellular carbohydrate content directly regulated rate of H_2 production. Synchronous culture with high capacity to photoproduce H_2 was shown to produce H_2 and O_2 in alternate periods.[185] The decrease in the capability of H_2 production by *Synechococcus* sp. Strain Miami BG 43511 due to exhaustion of cellular glycogen content was restored by the addition of various organic compounds.[186] Carbohydrates (glucose, fructose, sucrose, and maltose) were good substitutes, whereas pyruvate was the

only electron donor among the various organic acids tested. Xylose, arabinose, lactose, cellobiose, and dextrin did not act as electron donors. Ethanol and glycerol also supported H_2 production. The maximum rates of H_2 production achieved with 25 mmol of substrate were 1.11, 0.62, 0.50, 0.47, 0.37, and 0.39 μ mols/mg cell dry wt/ h for pyruvate, glucose, maltose, sucrose, fructose, and glycerol, respectively. The photo production of H_2 by synchronously grown cells of the same strain was also examined under high cell density conditions.[187] In a 25-ml reaction vessel, optimum H_2 production was achieved with 3 ml cell suspension containing 0.2 to 0.3 mg chlorophyll. H_2 and O_2 accumulated after 24 h were 7.4 and 3.7 ml, respectively. The energy conversion efficiency of photosynthetically active radiation was calculated to be 2.6%. Periodical replacement (24 h) of gas prolonged H_2 production up to 21 ml.

Although cyanobacteria are obligate photoautotrophs, some species could utilize simple organic compounds as electron donors for nitrogenase-mediated H_2 production.[186] *Synechococcus cedrorum* and a *Synechococcus* sp. OU 103 yielded appreciable amounts of biomass in the presence of ascorbate, glutamate, malate, pyruvate, succinate, and sucrose and *Synechococcus* sp., also in presence of sulfide. *S. cedrorum* produced an optimum amount of H_2 (11.8 mmol/vessel) in 10 ml medium containing 0.1% (w/w) malate, whereas *Synechococcus* sp. (10.3 mmol/vessel) in presence of 3 mM sulfide.[187] It was also tried to produce H_2 by immobilized mixed cultures of *S. cedrorum* and *Pseudomonas fluorescence* in alginate gel. However, inhibition of H_2 production was observed with the mixed culture.[188]

In a recent report Aoyama et al.[189] described production of H_2 (along with ethanol and organic acids) by *Spirulina platensis* NIES-46 under dark anaerobic conditions. The strain accumulated glycogen up to 50% of cell dry wt after photoautotrophic growth for 3 d in

a N_2 free medium. The cells at concentration of 1.624 mg dry wt/ml produce about 2 μ mol H_2/mg dry wt in 20 h alone. viz acetate (~3 μ mol), ethanol (~1 μ mol), formate (~0.8 μ mol) and lactate (~0.1 μ mol) during autofermentation under N_2 in dark conditions.

F. Simultaneous Production of Hydrogen and Oxychemicals

To improve the economy of microbial production of H_2, attempts at the production of both H_2 and oxychemicals of commercial value were also reported.

Vos et al.[190] reported efficiencies of 18 Enterobacteriaceae for the production of H_2 from glucose and formate. The resting cells of Klebseilla oxytoca ATCC 13182 released H_2 from formate with 100% efficiency, but at 5% efficiency from glucose considering the release of 2 mol H_2/mol of glucose. Heyndrickx et al.,[191] however, reported a higher efficiency (74%) of butanol-producing C. pasteurianum. They considered that glycerol could be an interesting substrate for H_2 production along with other byproducts by Enterobacteriaceae or saccharolytic Clostridia. C. butyricum converted glycerol to 1,3 propane-diol in addition to butyric acid, 2,3- butane-diol, formic acid, along with CO_2 and H_2.[192,193] Similarly, Klebsiella pneumoniae also converted glycerol into 1,3- propane-diol, acetic acid, ethanol, succinic acid, lactic acid, formic acid, CO_2, and H_2.[194–196] The fermentation of glycerol by the strains of C. butyricum LMG 1212t_2 and 1213t_1, and C. pasteurianum LMG 3285 was studied by Heynderickx et al.[191] In chemostatic culture C. butyricum LMG 1212t_2 converted 65% of glycerol to 1,3 -propane-diol without H_2 production. However, the addition of acetate at increasing concentrations resulted in fewer formations of propane-diol and more butyrate and H_2 production. C. pasteurianum LMG 3285 produced more than half of glycerol into n-butanol with significant production of H_2. The presence of acetate in the medium[197] did not affect the pattern of production of end-products.

Solomon et al.[198] analyzed material and available electron balance for H_2 production during anaerobic growth of Klebsiella pneumoniae DSM 2026 and C. butyricum DSM 5431 on glycerol. The specific rates of electron transfer to ethanol and H_2 formation were not growth-rate dependent in K. pneumoniae, but only in H_2 formation was growth rate independent in C. butyricum.

Recently, Woodward et al.[199] suggested an enzymatic method for the simultaneous production of molecular H_2 and gluconic acid from glucose. The method involved oxidation of glucose by glucose dehydrogenase (GDH) with generation of NADPH, which was used for stoichiometric reduction of H^+ by HD. GDH and HD were purified from Thermoplasma acidophilla and Pyrococcus furiosus, the two thermophilic Archae that grew optimally at 59°C and 100°C, respectively. Benemann[10] expressed the view, however, that the rate of gluconic acid accumulated (99% by weight of H_2 produced) was much higher than the present demand.

III. CONCLUSION

It is really wonderful to find a volume of work done on the biochemistry, enzymology, and process technology on the production of H_2 by microorganisms.

In general, production of H_2 by anaerobic, facultative, and photosynthetic microorganisms have been thoroughly investigated, each process having its pros and cons. Production of H_2 by anaerobic microorganisms has optimum stoichiometry (1:4, with glucose as substrate) compared with facultative anerobes (1:2), although the latter process is comparatively simpler than the former. Photosynthetic processes by cyanobacterium appear to have the highest potential in terms of stoichiometry and cost of substrate, yet require more complicated technology for commercial application. Unfortunately, each

process has not been vigorously evaluated in terms of the cost for commercialization. The authors believe that the production of H_2 by microbial processes has been researched extensively in search of a clean alternative to fossil fuels; however, the current situation is not severe enough to create much demand for the development of H_2 biotechnology. The future of H_2 biotechnology based on the present knowledge is really at a junction point, and the research would likely be dictated by the global situation on the stock of the fossil fuel and the extent of environmental pollution it causes.

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