

# Integrative Biological Hydrogen Production: An Overview

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**Abstract** Biological hydrogen (H<sub>2</sub>) production by dark and photo-fermentative organisms is a promising area of research for generating bioenergy. A large number of organisms have been widely studied for producing H<sub>2</sub> from diverse feeds, both as pure and as mixed cultures. However, their H<sub>2</sub> producing efficiencies have been found to vary (from 3 to 8 mol/mol hexose) with physiological conditions, type of organisms and composition of feed (starchy waste from sweet potato, wheat, cassava and algal biomass). The present review deals with the possibilities of enhancing H<sub>2</sub> production by integrating metabolic pathways of different organisms—dark fermentative bacteria (from cattle dung, activated sludge, *Caldicellulosiruptor*, *Clostridium*, *Enterobacter*, *Lactobacillus*, and *Vibrio*) and photo-fermentative bacteria (such as *Rhodobacter*, *Rhodobium* and *Rhodospseudomonas*). The emphasis has been laid on systems which are driven by undefined dark-fermentative cultures in combination with pure photo-fermentative bacterial cultures using biowaste as feed. Such an integrative approach may prove suitable for commercial applications on a large scale.

**Keywords** Biowaste · Dark-fermentation · Hydrogen · Mixed culture · Photo-fermentation

## Introduction

Hydrogen (H<sub>2</sub>) has been recognized as fuel for the future due to its high efficiency (122 kJ/g) and eco-friendly nature in

comparison to fossil fuels [1, 2]. Biological H<sub>2</sub> production (BHP) process has been widely studied under dark- and photo-fermentative conditions. With these approaches the yields of H<sub>2</sub> have been quite low in comparison to the theoretically achievable values of 4 and 8 mol/mol of glucose under dark and photo-fermentative conditions, respectively [2–7]. Quite a few research efforts have been made to overcome the limitations of these processes. It has been realized that in order to recover maximum H<sub>2</sub> from the organic matter, it is necessary to further use the end-products of the dark fermentative process, especially volatile fatty acids (VFA). It is possible to convert VFAs to H<sub>2</sub> by photosynthetic bacteria. The potential of exploiting these processes in various combinations have been reviewed to some extent [7–10]. However a large number of hurdles still seem to persist such as: (i) in the dark-fermentative process—(a) relatively lower H<sub>2</sub> yield (b) the need for strict anaerobic conditions for high H<sub>2</sub> producers, and (c) thermodynamic instability of the process at higher H<sub>2</sub> concentrations, and (ii) during the photo-fermentative process—(a) sensitivity of the H<sub>2</sub> production process to nitrogen content of the feed (b) effect to light intensity and duration of radiation under outdoor (sunlight) and indoor (artificial light sources) conditions, and (c) types of bioreactors required for H<sub>2</sub> production [2, 11–14].

High cost of the feed and operational conditions is the major limiting factor of BHP. Most basic studies have been carried out on simple and complex sugars as feed material [15–21]. For circumventing the issues related to cost of the feed, biowastes of diverse origins especially agricultural, food and fruit processing industries, and those of municipal markets have been suggested as cheap and renewable alternatives [2, 22–28]. Although, the amount of H<sub>2</sub> generated from different biowastes encourages one to pursue this route however, it demands quite a bit of optimization at

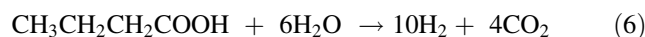
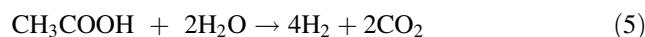
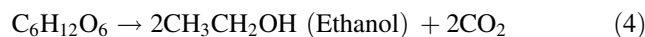
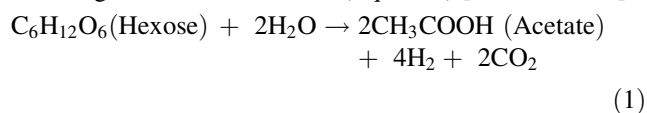
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different stages [29–34]. Instead of dwelling on optimization efforts being made on individual parameters of BHP process, an emerging proposal is to combine the dark- and photo-fermentative H<sub>2</sub> producing organisms [7, 10, 35, 36]. The efforts in this direction have been targeted on the following combinations: (i) using defined dark- and photo-fermentative H<sub>2</sub> producing organisms in a sequential manner in two independent stages (ii) using undefined dark-fermentative H<sub>2</sub>-producers along with defined photosynthetic organisms in two stages (iii) using the two types of BHP processes into a single stage, and (iv) using effluent from a dark-fermentative process (not necessarily a H<sub>2</sub> production reactor) and exploiting photo-fermentative bacteria for their H<sub>2</sub> producing abilities [33, 37–48]. In our recent efforts, we have emphasized only on using defined bacterial cultures in a sequential manner and evaluate it with respect to their individual H<sub>2</sub> producing abilities from pure substrates and biowastes [7]. In the present work, we are concentrating our efforts on studies conducted using undefined dark fermentative H<sub>2</sub> producing culture combinations and exploitation of effluent from dark-fermentative process by photosynthetic organisms using biowaste as feed.

## Biological Hydrogen Production

### Integrative Two Stage Dark- and Photo-Fermentative Sequential Hydrogen Production

The physiology and metabolic activities of bacteria vary significantly under dark- and photo-fermentative conditions. The efficiency depends primarily on the types of enzymes involved in H<sub>2</sub> evolution. Under dark-fermentative conditions, hydrogenase and nitrogenase are the major enzymes responsible for this process [2, 49]. In the overall conversion of feed to H<sub>2</sub>, a few intermediates are also generated, such as VFAs and alcohols. The efficiency of the dark-fermentative H<sub>2</sub> evolution process is governed by VFAs (Eqs. 1–4), such that acetic acid generation can lead to an additional 4 mol of H<sub>2</sub> whereas butyric acid is expected to generate 2 mol of H<sub>2</sub>/mol of substrate. Lactic acid and ethanol are considered to be counter-productive to H<sub>2</sub> evolution process [2, 28]. The intermediates of the dark-fermentative BHP, such as acetic and butyric acid can be taken up by photosynthetic organisms to generate additional H<sub>2</sub> (Eqs. 5–6) [45, 46, 50, 51].



Using the organisms present in activated sludge enriched for dark-fermentative H<sub>2</sub>-producers, along with photosynthetic organisms such as *Rhodobacter sphaeroides*, *Rhodospseudomonas palustris* and undefined photosynthetic bacteria, it has been possible to achieve 2.86–6.07 mol H<sub>2</sub>/mol hexose [34, 52], over an incubation period ranging from 1 to 6 days of dark-fermentation followed by 5–14 days of photo-fermentative phase [53, 54]. In most of the cases, the temperature of 31–37 °C has been found to be optimal during the dark phase and 30 °C during the light phase (Table 1). In these cases, starchy wastes have been employed, which had originated from wheat, rice and cassava (Table 1). In other studies, cattle dung, dairy manure and mixed cultures in combinations with *Rhodobacter capsulatus*, *R. palustris*, and *R. sphaeroides*, and their combinations have been shown to yield 3.40–7.15 mol H<sub>2</sub>/mol hexose [33, 38, 43, 47, 55, 56]. In these cases, starchy wastes, cheese whey and water hyacinth have been fermented for quite long periods 2–10 days of the dark phase followed by 11–21 days of the light period and exceptionally it was 90/100 days under repeated batch culture [47]. In a few other combinations of dark and photosynthetic bacteria, *Caldicellulosiruptor*, *Clostridium*, *Klebsiella*, *Lactobacillus* and *Thermotoga* in association with *R. capsulatus*, *R. sphaeroides*, *Rhodobium marinum* and *R. palustris* have been used for H<sub>2</sub> production (Table 1). These integrative approaches of two stage H<sub>2</sub> production have proved effective as most of them have lead to yields up to 7.2 mol/mol hexose [30]. In dark-fermentative BHP, the H<sub>2</sub> yields are quite low in most cases and exceptionally it is possible to achieve a value of 3.8 mol H<sub>2</sub>/mol hexose [57]. In contrast, the two stage integrative approach is much more effective and exceptionally only it falls around 2.8–3.9 mol/mol hexose [45, 51, 58]. A summary of the results of yields of ≥7.0 mol H<sub>2</sub>/mol hexose reveals that it has been achieved with combinations such as (i) mixed culture—*R. palustris* and water hyacinth (10 g/l) [33], (ii) *Clostridium butyricum*—*R. sphaeroides*—algal biomass (starch at the rate of 5 g/l) [31], (iii) *C. butyricum* and *Enterobacter aerogenes*—*R. sphaeroides*/*Rhodobacter* sp. and sweet potato starch (5–10 g/l) [30, 59].

A perusal of Table 1 allows us to draw a few conclusions on the significance of the roles of photosynthetic organisms in influencing H<sub>2</sub> yields in the integrative BHP process. Here, it can be observed that photo-fermentative organisms can utilize different biowastes to produce high H<sub>2</sub> yields—(i) *R. sphaeroides* could evolve 3.81–8.30 mol H<sub>2</sub>/mol hexose (ii) *R. capsulatus* yielded 3.90–6.85 mol H<sub>2</sub>/mol hexose, and (iii) *R. palustris* was also effective in

**Table 1** Integration of dark- and photo-fermentative bacteria in a sequential two stage hydrogen production from biowastes

Organisms	Substrate (concentration: g/l)		Process parameters (in dark/light phase)				H <sub>2</sub> yield (mol/mol hexose)	Reference
	Photo-fermentative		Reactor capacity (l)	pH	Temp. (°C)	IP <sup>a</sup> (days)		
Activated sludge	<i>Rhodobacter sphaeroides</i>	Wheat starch (20.0)	0.31/0.31	6.8/7.1	37/30	3/8	Batch/Batch	[20]
	Mixed strains ( <i>R. sphaeroides</i> )	Wheat starch (20.0)	0.31/0.31	6.8/7.5	37/30	– <sup>d</sup> /14	Batch/Batch	[53]
	<i>Rhodospseudomonas palustris</i>	Cassava starch (10.0)	0.30/0.30	7.0/7.0	35/30	6/5	Batch/Batch	[52]
	Mixed PSB <sup>a</sup>	Rice straw (50.0)	0.30/0.10	6.5/7.0	35/30	1/–	Batch/Batch	[54]
Cattle dung compost	<i>R. sphaeroides</i>	Cassava starch (10.4)	0.30/0.10	6.3/7.0	31/30	3/8	Batch/Batch	[34]
	<i>R. sphaeroides</i>	Cassava starch (18.0)	0.038/0.038	6.8/7.0	37/30	4/8	Batch/Batch	[38]
	<i>R. sphaeroides</i>	Food waste (20.0)	0.038/0.038	6.8/7.0	37/30	4/8	Batch/Batch	[38]
Dairy manure microflora	<i>R. sphaeroides</i>	Comcob (Sugar, 10.0)	0.60–2.5/0.32	7.0/7.0	36/35	–/12	Batch and CSTR <sup>b</sup> /Batch	[43]
	<i>R. palustris</i>	Cheese whey (COD, 30.0)	1.0/0.25	7.5/6.9	55/31	–/21	CSTR (HRT <sup>c</sup> –24h)/Batch	[55]
<i>Clostridium butyricum</i>	<i>R. sphaeroides</i>	Water hyacinth (10.0)	0.30/0.30	7.0/7.0	35/30	2–5/11	Batch/Batch	[33]
	<i>R. palustris</i>	Potato starch (50.0–100.0)	0.25/0.30	6.8/7.0	37/28	10–12/25	Batch/Batch	[56]
	<i>R. sphaeroides</i>	Starch (20.0)	0.50/100 cm <sup>3</sup> (dimensions)	6.8/6.4	37/28	90/100	Batch <sup>b</sup> /Batch <sup>b</sup>	[47]
	<i>R. palustris</i>	Algal biomass (Starch, 5.0)	0.15/0.15	6.8/7.0	37/30	2/15	Batch/Batch	[31]
<i>C. butyricum</i> and <i>Enterobacter aerogenes</i>	<i>R. palustris</i>	Starch (17.0)	0.20/1.00	7.5/7.1	37/32	6–32/–	Batch/Batch and CSTR/CSTR (HRT–12/24h)	[37]
	<i>Rhodobacter</i> sp.	Starch (5.0)	TT <sup>b</sup> /TT	7.0/6.5	30/30	–/–	Batch	[68]
	<i>R. sphaeroides</i>	Sweet potato starch (Starch, 5.0)	0.25/TT	5.3/7.5	37/35	10/30	Batch <sup>b</sup> /Batch <sup>b</sup>	[59]
	<i>Rhodobacter</i> sp.	Sweet potato starch (Starch, 10.0)	0.25/TT	5.3/7.5	37/35	13/30	Batch <sup>b</sup> /Batch <sup>b</sup>	[30]
<i>Clostridium acetobutylicum</i> and <i>Escherichia coli</i>	<i>R. capsulatus</i>	Date palm fruits and sucrose (5.0)	2.00/2.00	7.3/7.0	30/30	3/7	Batch/Batch	[58]
	<i>R. capsulatus</i>	<i>Miscanthus</i> (Sugar, 10.0)	2.00/0.105	7.0/6.5	80/30	3/10	Batch/Batch	[57, 60]
<i>Thermotoga neapolitana</i>	<i>R. capsulatus</i>	Potato steam peels (Sugar, 15.0)	2.00/0.055	6.8/6.4	72/30	–/–	Batch/Batch	[51]
	<i>R. capsulatus</i>	Sugar beet molasses (Sucrose, 15.0)	1.00/0.055	6.9/6.6	72/30	3/12	Batch/Batch	[32, 46]
<i>Caldwellulosiraptor saccharolyticus</i>	<i>R. capsulatus</i>	Potato steam peels (Sugar, 15.0)	1.00/0.055	6.9/6.7	72/30	3/7	Batch/Batch	[32]
	<i>R. capsulatus</i>	Potato steam peels (Sugar, 15.0)	2.00/0.055	6.8/6.4	72/30	–/–	Batch/Batch	[51]
<i>Klebsiella oxytoca</i>	<i>R. capsulatus</i> , <i>R. palustris</i> and <i>R. sphaeroides</i>	Potato steam peels (Sugar, 15.0)	2.0/0.055	6.4/6.4	72/30	–/6	Batch/Batch	[45]
	<i>R. palustris</i>	Sugarcane bagasse (50.0)	0.14/0.14	7.0/7.0	37/30	–/–	Batch/Batch	[44]
<i>Lactobacillus amylovorus</i>	<i>Rhodobium marinum</i>	Algal biomass (Starch, 4.05)	0.07/0.07	7.0/6.5	30/30	6/6	Batch/Batch	[29]

<sup>a</sup> Photo synthetic bacteria  
<sup>b</sup> Test tube with dimension of 2.4 × 20 cm<sup>2</sup>  
<sup>c</sup> Incubation period  
<sup>d</sup> Values not given  
<sup>e</sup> Continuous stirred tank reactor  
<sup>f</sup> Hydraulic retention time  
<sup>g</sup> Both in batch and repeated batch cultures  
<sup>h</sup> Values converted from the original data

generating up to 7.15 mol H<sub>2</sub>/mol hexose [31–33, 53, 58]. In view of the effective working of the photosynthetic partners in the integrated BHP process, the observed variations in H<sub>2</sub> yields can be assigned to the dark-fermentative H<sub>2</sub>-producers. Dark-fermentative bacteria present in the activated sludge were relatively less effective in producing H<sub>2</sub> in comparison to those present in cattle dung. Among the defined dark fermentative bacteria, *C. butyricum* alone or in association with *E. aerogenes* was quite consistent in yielding 7–8 mol H<sub>2</sub>/mol hexose, along with *R. sphaeroides* as the photo-fermentative partner [31, 59]. H<sub>2</sub> yields did not vary when *R. capsulatus* was used in association with a wide range of dark-fermentative H<sub>2</sub>-producers [47, 51, 57, 58, 60]. In contrast to *R. sphaeroides*, the combination of *C. butyricum* and *R. palustris* did not prove to be the most effective H<sub>2</sub>-producing culture combination [37].

#### Integrative Single Stage Dark and Photo-Fermentative Hydrogen Production

In contrast to subjecting feed material to dark- and photo-fermentative bacteria under two different sets of conditions, attempts have been made to combine the two (Table 2). Combination of activated sludge (as source of dark-fermentative H<sub>2</sub>-producers) with *R. sphaeroides* has resulted in H<sub>2</sub> yield of 0.3–3.4 mol/mol hexose [39, 61–63]. The variation in H<sub>2</sub> yields could be assigned to differences in substrate concentration, inoculum ratios, light intensities, etc. [39, 63]. In most of the reports, batch and fed-batch mode of reactors have been employed. The best results of 3.4 mol H<sub>2</sub>/mol hexose were reported when wheat starch was used at the rate of 5 g/l with an inoculum ratio of 1:3 (of dark/photo-fermentative bacteria) in continuous mode (periodic feed) [63].

In other experiments, low H<sub>2</sub> yields in the range of 1.05–1.16 mol/mol hexose were recorded on substituting *R. sphaeroides* with *Rhodobacter* sp. and *R. palustris* combination, along with activated sludge and wheat starch (as feed) [64, 65] and quite high yield of 2.76 mol/mol hexose with pure culture [66]. It allowed one to conclude the superiority of *R. sphaeroides* as a photo-fermentative partner. The high H<sub>2</sub> yielding capacity of *R. sphaeroides* was negatively affected when combined with *Clostridium beijerinckii* as the dark-fermentative partner—resulting in low H<sub>2</sub> yield of 0.6 mol/mol hexose [41]. *R. marium* proved to be an effective H<sub>2</sub>-producer, which resulted in high yields of 7.3 mol/mol hexose with *Lactobacillus amylovorus* and 6.2 mol/mol hexose with *Vibrio fluvialis* [29, 67]. Incidentally, in spite of being such highly effective H<sub>2</sub>-producers, *L. amylovorus* and *V. fluvialis* have not been pursued since their initial reports.

#### Perspectives

Among the different worries which loom large are the pollution due to burning of fossil fuels and their limited resources. Although biohydrogen has been identified as a clean alternative to ever polluting fossil fuels, however, in order to establish biohydrogen as a non-polluting energy carrier it is imperative to carry out innovative research. At present, the struggle is on to look for cheap sources of feed and robust microbes for commercial scale H<sub>2</sub> production. The need stems from the fact that BHP is regarded as inefficient due to low yields. Theoretically 12 mol of H<sub>2</sub> can be generated from each mol of glucose. However, in practice, H<sub>2</sub> yields are stagnant, such that a maximum of 3.8 mol/mol glucose has been shown as the achievable limit with either dark- or photo-fermentative routes by a limited number of bacteria. It was however realized quite soon that H<sub>2</sub> yields can be enhanced by combining the two metabolic routes. Here, VFAs especially acetic acid and butyric acid generated as the end products of dark fermentative H<sub>2</sub>-production process can be subjected to photo-fermentative bacteria. Theoretically, acetic acid can be converted to generate 4 mol of H<sub>2</sub> [50]. Such that a H<sub>2</sub> yield of 12 mol/mole glucose can be achieved by employing an integrative approach—dark followed by photo-fermentation [7]. The need is to optimize the various process parameters and thus improve the efficiency of the organisms. Since, bacteria exist largely as complex communities, they create conditions such that ecological selection persists and the most productive system prevails. Taking advantage of the abilities of the bacteria to occur as mixed cultures and as consortia, it is desirable to select bacteria which are compatible to each other and exploit their natural abilities to accomplish our purpose. Facultative anaerobes such as *Bacillus* and *Enterobacter* have abilities to produce H<sub>2</sub> in quantities which are quite comparable to those produced by strict anaerobic (*Clostridium*). They however offer additional advantages in terms of their abilities to survive in the presence of O<sub>2</sub> during the initial stage of anaerobic biodegradation and produce H<sub>2</sub> efficiently. They also offer an added feature by quenching O<sub>2</sub> in cases where *Clostridium* may be the associated H<sub>2</sub>-producer [68, 69]. In case of photo-fermentation, light intensity is a major requirement for most metabolic activities. During photo-fermentative BHP, nitrogenase enzyme requires energy for the H<sub>2</sub> production, which is provided by the light energy conversion to ATP [70]. It has been shown that increase in the light energy does enhance BHP [39], although exceptionally it may not prove effective [71]. We can design complex communities consisting of robust and self stabilizing populations. This syntrophic association must be managed for the sustainable development. It is envisaged that the feasibility of these two stage processes

**Table 2** Integration of dark- and photo-fermentative bacteria in a single stage hydrogen production from biowastes

Organisms	Substrate (concentration: g/l)	Process parameters				H <sub>2</sub> Yield (mol/mol hexose)	References			
		Inoculum ratio <sup>a</sup>	Light intensity <sup>c</sup>	Reactor capacity (L)	Temp. (°C)					
Activated sludge + <i>Rhodobacter sphaeroides</i>	Wheat starch (5.0)	1:3	5,000 lux	0.25	7.0	30	– <sup>i</sup>	Continuous <sup>k</sup> (HRT <sup>l</sup> –8)	3.40	[63]
	Wheat starch (5.95)	1:2	10,000 lux	0.31	7.5	30	8	Batch	1.45	[39]
	Wheat starch (10.0)	1:2	5,000 lux	2.00	7.5	30	10	Fed-batch	1.32°	[61]
	Wheat starch (12.8)	1:2	6,000 lux	0.31	7.5	30	12	Batch	0.28–0.36°	[62]
	Wheat starch (2.5)	1:7	9,500 lux	0.31	7.5	30	8	Batch	1.05	[64]
Activated sludge + <i>Rhodobacter</i> sp. and <i>Rhodospirillum rubrum</i>	Wheat starch (5.0)	1:7	9,500 lux	0.31	7.3	30	13	Batch	1.16	[65]
	Wheat starch (20.0)	1:2	9,500 lux	2.00	7.5	30	11	Combined fed-batch	0.43°	[42]
	Cassava starch (COD, 20)	1:1	6,000 <sup>d</sup> candela/m <sup>2</sup>	0.075	7.2	30	–	Batch	340 <sup>p</sup>	[71]
<i>Lactobacillus amylovorus</i> + <i>Rhodobium marinum</i>	Algal biomass (Starch, 4.05)	0.5:0.6	330 W/m <sup>2</sup>	0.07	6.5	30	16	Batch	7.30	[29]
<i>Clostridium beijerinikii</i> + <i>R. sphaeroides</i>	Wheat starch (5.0)	1:3.9	10,000 lux	7.63	7.3	32	–	AHB <sup>m</sup> (HRT-6)	0.60	[41]
	Wheat starch (12.8)	1:2	6,000 lux	0.31	7.5	30	12	Batch	0.12–0.15°	[62]
<i>Clostridium butyricum</i> + <i>Rhodobacter</i> sp.	Starch (5.0)	2:3	5,000 lux	TT <sup>f</sup>	6.5	30	8–40 <sup>j</sup>	Batch and fed-batch <sup>n</sup>	4.50–6.60	[68]
	Sugar cane effluent (Sugar, 7.9)	1:1:1 <sup>b</sup>	NA <sup>c</sup>	10–100 <sup>g</sup>	7.0	37	2	Batch	2.76	[66]
<i>Vibrio fluvialis</i> + <i>Rhodobium marinum</i>	Algal biomass (Starch, 4.05)	2:1	330 W/m <sup>2</sup>	0.07	7.0	30	9	Batch	6.20	[67]

<sup>a</sup> Dark/light organisms

<sup>b</sup> Co-cultures

<sup>c</sup> Continuous light

<sup>d</sup> Dark and light periods of 12 h each also

<sup>e</sup> Not applicable

<sup>f</sup> Test tube with dimension of 2.4 × 20 cm<sup>2</sup>

<sup>g</sup> Reactor dimensions in m<sup>3</sup>

<sup>h</sup> Incubation period in days

<sup>i</sup> Values not given

<sup>j</sup> Different sets of experiment

<sup>k</sup> Periodic feed

<sup>l</sup> Hydraulic retention time in days

<sup>m</sup> Annular-hybrid bioreactor

<sup>n</sup> Repeated

<sup>o</sup> Values converted from the original data

<sup>p</sup> ml H<sub>2</sub>/g COD



can be established by combining it with microalgae photosynthesis processes, which is likely to enhance overall H<sub>2</sub> production by utilizing CO<sub>2</sub> produced in the previous stages [37]. From a commercial point of view, it may be necessary to integrate other processes such as bioplastic and methane production in it [4, 7, 17, 72–75].

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