Indian J Microbiol (Jan–Mar 2018) 58(1):8–18
https://doi.org/10.1007/s12088-017-0678-9

REVIEW ARTICLE

Nanoparticles in Biological Hydrogen Production: An Overview

Sanjay K. S. Patel[1,2] · **Jung-Kul Lee**[1] · **Vipin C. Kalia**[2]

Received: 4 September 2017 / Accepted: 19 September 2017 / Published online: 22 September 2017

Abstract Biological hydrogen (H_2) production enhancement through the use of nanoparticles (NPs) supplement in the media is being recognized as a promising approach. The NPs, including those of metal and metal oxides have shown a significant improvement in the BHP. A number of organisms as pure or mixed cultures can produce H_2 in presence of NPs from pure sugars and biowaste as a feed. However, their H_2 production efficiencies have been found to vary significantly with the type of NPs and their concentration. In this review article, the potential role of NPs in the enhancement of H_2 production has been assessed in dark- and photo-fermentative organisms using sugars and biowaste materials as feed. Further, the integrative approaches for commercial applications of NPs in BHP have been discussed.

Keywords Biowaste · Dark-fermentation · Hydrogen · Mixed microbial culture · Nanoparticles · Pure culture

Introduction

The worldwide dependence of fossil fuels as major source of energy has lead to profound negative influence on the natural environment and human health. The production of biofuels to reduce the usage of fossil fuels and if possible replace them has gained considerable attention to overcome the limitations of their fixed natural resources. The major worry is the likely hood of exhausting coal and petroleum reservoirs within the next 150–200 years [1–5]. A few potentially ecofriendly approaches have been evaluated to produce biofuels in future, which include biomethanol, biohydrogen (H_2), biomethane and biodiesel [5–11]. H_2 as a renewable source of energy is considered to be the cleanest and most energy efficient (122 kJ/g). The biological H_2 production (BHP) at ambient physiological conditions is the most obvious and viable approach over energy intensive conventional chemical or electrochemical processes [2, 12]. The latent advantage of BHP is the potential of obtaining it from biological wastes rich in organic matter. Here, large quantum of waste generated from diverse sources especially food industry and agricultural practices seems to be a viable feedstock for BHP. It also has the advantage of effective waste management, which thus prevents further environmental pollution [1, 3, 13]. BHP can be achieved by various methods, including biophotolysis, photo-fermentation, dark-fermentation and microbial electrolysis. Among these processes, H_2 production has been widely reported by phylogenetically diverse dark- and photo-fermentative microorganisms [2, 9]. This search for novel H_2 producers has been aided by screening of strains isolated from the diverse environmental habitats and comparative genomic techniques. These studies have enabled identification of strains with high H_2 producing potential [14, 15]. Microbial H_2 production is influences by physiological factors, such as pH, feed, temperature, and type of inoculum [9, 16]. The search for assessing efficient H_2 producers has been facilitated by analyzing the metabolites produced during fermentative H_2 production. Overall, the dark-fermentative H_2 production showed advantages of high production rate and light

✉ Sanjay K. S. Patel
sanjaykspatel@gmail.com; sanjaykspatel@yahoo.co.in

[1] Department of Chemical Engineering, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul 143-701, Korea

[2] Microbial Biotechnology and Genomics, CSIR–Institute of Genomics and Integrative Biology (IGIB), Delhi University Campus, Mall Road, Delhi 110007, India

independent process as compared with photo-fermentation [3]. Also, to improve the dark-fermentative H_2 production, the genetic engineering has been performed using strains, including *Clostridum* spp. (*C. acetobutyricum*, *C. beijerinkii*, *C. butyricum*, *C. paraputrificu*, *C. saccharoperbutylacetonicum*, *C. thermolacticum*, *C. tyrobutyricum* and *Clostridium* sp.), *Escherichia coli* and *Enterobacter* spp. (*E. aerogenes*, *E. cloacae*, *E. asburiae* and *Enterobacter* sp.) [17, 18].

The significant developments in the area of nanotechnology accelerating have improved their potential applications in improving the biological processes. This has been specially aided by the availability of nanostructured materials—nanoparticles (NPs), which have suitable physical and chemical properties [19–24]. The bioprocess for generating H_2 by microorganisms occurs through either disposal of excess reducing equivalents or as nitrogen fixation byproduct [25]. These reactions are catalyzed by hydrogenase and nitrogensae enzymes in the dark- and photo-fermentative organisms. Hydrogenase enzymes are classified into different groups based on the type of metal atom present in their active site, which are thus categorized as [NiFe]-, [FeFe]- and [Fe]-hydrogenases [25]. Both pure or mixed culture H_2 production is greatly influenced by the presence of the iron (Fe) and nickel (Ni) metal ions due to their involvement in enhancing the activity of hydrogenase. Recently, to overcome the limitation of low H_2 production under dark-fermentative process, the use metal and metal oxide NPs have been suggested via: (1) enhanced intracellular electron transfer, (2) assisting the H_2 producing [NiFe]- or [FeFe]- enzymes, and (3) their anti-microbial properties to enrich selective H_2 produces in the mixed culture [26, 27]. However, the biocompatibility of metal particles is a primary concern for the H_2 producers. Further, the use of biologically synthesized NPs appeared to be a more promising approach than the physical and chemically synthesized NPs [28–30]. In this article, we have described the potential of different types of NPs and their compositions on the BHP by pure and mixed cultures using sugars and biowastes as a feed. All those studies involving composite particles for the BHP process have not been discussed here.

Biohydrogen Production

The H_2 production efficiency or yield during dark- and photo-fermentative depends on the type of organisms and the metabolites produced as end by-products [2, 3, 31]. Pure sugars are widely used as feed for the H_2 production by different organisms, the well known ones include *Bacillus*, *Caldicellulosiruptor*, *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, *Escherichia*, *Rhodobacter*, *Rhodopsedomonas* and *Thrmotoga*, under either mesophilic or thermophilic conditions [9, 32–36]. These organisms result in H_2 yields of 0.6–3.98 mol/mol of hexose [9, 35]. The complete utilization of hexose sugars result in maximum production of 4 and 8 mol via dark- followed with photo-fermentative processes respectively, finally leading to 12 mol H_2/mol of hexose [3]. The H_2 production through dark-fermentative process showed only utilization of one-third of substrate utilization. Thus, relative low energy conversion efficiency of the dark-fermentative process lead to practical limitations at commercial scale. In addition to this comes the high cost of feed turns out to be a major challenge. Still, the maximum H_2 production equivalent to stoichiometric yield of 12 mol H_2/mol of hexose is not feasible even by best combination of H_2 producers of dark- and photo-fermentative processes [3, 4, 9]. The synthetic enzymatic cascade system has shown the feasibility of achieving the H_2 production close to the maximum theoretical yield by using sucrose as feed [37]. Thus, the high cost of purified enzymes used in cascaded or cocktails system and their low stability is also a major hurdle for commercial level of production. There is a primary concern for the development of efficient BHP system using biowaste as a feed as their chemical nature is quite complex. Various pretreatment methods, including chemical, physical and microbial have been adopted to enhance the hydrolysis of biomass for improving H_2 production [34, 38–41]. Among these, microbial pre-treatment of biomass seems an economically effective approach for H_2 production, since these operate even under unsterilized conditions [34, 40, 41].

Influence of Nanoparticles on Biohydrogen Production

The use of NPs has been increasing significantly for applications, such as protein immobilization, biosensors and biofuels production [19, 22, 30, 42, 43]. Biosensors are used for enhancing electron transfer to acceptors. NPs can also significantly influence the microbial metabolic activity for H_2 production through similar phenomenon under aerobic condition by efficient transfer of electrons [44]. Thus, a positive effect of various NPs, including silver (Ag), gold (Au), Copper (Cu), Fe, Ni, Palladium (Pd), Silica (SiO_2), Titanium (Ti), activated carbon, carbon nano-tubes (CNTs) and composite were observed on BHP [19, 29, 30, 44–51]. Briefly, these NPs might be stimulating BHP by their surface and quantum size effect [49]. As surface effect, smaller the size of NPs larger specific surface area, which thus enables strong ability to adsorb electrons. The extent of the quantum size is directly co-related with the rate of electron transfer between NPs and

enzyme molecules such as hydrogenase, which is known to catalyze the conversion of H_2 to proton and vice versa, either to act as electron sinks or deliver reducing power from H_2 oxidation, as follow: $H_2 \leftrightarrow 2H^+ + 2e^-$ [49]. Overall, the influence of individual and mixtures of NPs on the BHP yield by different organism has been presented in Table 1.

Inorganic Particles

Copper, Silver, Gold and Palladium

The synthesis of various types of NPs has been demonstrated using physical, chemical and biological processes for their application in the BHP [19, 29, 30, 51, 52]. Among these synthetic approaches, green synthesis of NPs through biological method using the plant leaf extract has been suggested as a suitable alternative approach over harsh conditions adopted in physical and chemical NPs synthesis processes [29, 30, 52]. Cu NPs (CuNPs) showed a negative influence on the H_2 production by both *C. acetobutyricum* NCIM 2337 and *E. cloacae* 811101 strains over a concentration range of 2.5–12.5 mg/l using glucose as feed [30]. At the lower concentration of 2.5 mg/l of CuNPs, a 3.5 and 2.9% reduction in H_2 yield was recorede as compared to controls, where the yields were 1.74 and 1.44 mol H_2/mol hexose, respectively. A significant reduction of 56.9 and 72.2% in the H_2 yeild was observed at the concentration of 12.5 mg/l, respectively. These lower H_2 yields were associated with inhibitory effect of CuNPs on *C. acetobutylicum* NCIM 2337 and *E. cloacae* 811101, which were close to their maximum inhibitory concentrations of 13.3 and 15.2 mg/l, respectively [30]. In addition, the lower acetate to butyrate ratio at the higher concentration of CuNPs justified the decrease in the yield. In general, the H_2 yield was positively associated with high ratio of acetate and butyrate, and lower production was negatively associated with production of propionate and alcohols [2, 9]. Overall, CuNPs exhibited more inhibitory effect on the BHP yield by both *C. acetobutylicum* NCIM 2337 and *E. cloacae* 811101 as compared with Cu^{2+} ions [30], which might be directly associated with the high antimicrobial activity of CuNPs. On the contrary, the CuNPs encapsulate SiO_2 composite was found beneficial in improving H_2 yield from 0.92 to 1.01 mol/mol hexose by *C. butyricum* CWBI1009 [44]. These results suggested that CuNPs concentration below to 2.5 mg/l or encapsulation in porous matrix can be used to enhance the H_2 production by regulating its antimicrobial activity [30, 44].

Silver NPs (AgNPs) exhibited wide range of applications due their unique characteristics, including support for immobilization of protein, electronics, food and medical industries [28, 43, 53]. In spite of their known anti-microbial activity, Zho et al. [51] showed the effective use of AgNPs in H_2 production from glucose by mixed culture dominated by *C. butyricum*. Here, an increase in the concentration AgNPs from 10 to 20 nM resulted in an enhancement in the H_2 production thereafter it was quite consistent till concentration up to 200 nM. Overall, the maximum production of 2.48 mol H_2/mol glucose was observed at the AgNPs concentration of 20 nM with an enhancement of 67.6% in the yield as compared to control (1.48 mol H_2/mol glucose). The production of metabolic intermediates such as ethanol, acetate, butyrate, valerate, and propionate was significantly altered in the presence of AgNPs. Here, the higher H_2 production was associated with an increased amount of acetate and butyrate and significant decrease of ethanol, propionate and valerate in the presence AgNPs than the control. Interestingly, the high AgNPs concentration stimulated higher cell biomass production rate and reduced the lag phase for H_2 production [51].

Gold NPs (AuNPs) are also known to enhance catalytic properties in chemical processes (such as hydrogenation and oxidation) and biological application to improve enzyme activity and immobilization [49]. Zhang and Shen [49] have established the role of AuNPs in improving the fermentative BHP. With synthetic wastewater containing sucrose as a feed, anaerobic culture resulted in 62.3% higher yield (2.28 mol H_2/mol hexose) than those to the control (1.38 mol H_2/mol hexose) at the 5 nM of AuNPs. Remarkably, the H_2 production enhancement was reciprocally dependent on the concentration of AuNPs. AuNPs also altered the metabolites concentration during the H_2 production process [49, 51]. The high ratio of acetate to butyrate and low production of ethanol in the presence of AuNPs is associated with the significant increase in the H_2 production. This study suggested that the use of pre-heated anaerobic culture dominated with *C. butyricum* as inoculum and AuNPs provided a suitable approach for efficient H_2 production from sucrose [49]. Similarly, the feasibility of H_2 production in a single chamber reactor using electrochemically active biofilm (EAB) and acetate as a substrate by anaerobic sludge in the presence of AuNPs was demonstrated [52]. EAB developed on stainless steel mesh and carbon paper with anaerobic sludge exhibited 44.0% H_2 production of maximum theoretical yield from acetate as feed at AuNPs concentrations of 1 mM. Interestingly, no H_2 production was observed by EAB in the absence of AuNPs [52].

Mohanraj et al. [29] briefly evaluated the effect of phytogenic synthesized palladium NPs (PdNPs) using *Cortandrum sattvum* leaf extract on the H_2 production by *E. cloacae* 811101 and mixed culture from glucose as feed. At 5.0 mg/l PdNPs, 0.6 and 6.4% higher yield with

Table 1 Biological hydrogen production in the presence of different types of inorganic and organic nano-particles

Nano-particles		Organisms	Feed	Process parameters				Yield[c]	H_2 yield efficiency (%)	Refereces
Composition	Conc. (mg/l)[a]			Mode	WV (L)	pH	Temp. (°C)			
Inorganic										
Ag	20.0[b]	Mixed culture	Glucose	Batch	0.08	8.5	35	2.48	67.6	[51]
Au	1.0 mM	Anaerobic sludge	Acetate	EAB	0.20	7.2	35	1.76	na[i]	[52]
	5.0[b]	Anaerobic culture	Wastewater	Batch	0.08	7.2	35	2.24	50.0	[49]
Cu	2.5	*Clostridium acetobutylicum* NCIM 2337	Glucose	Batch	0.20	7.0	37	1.39	3.5[j]	[30]
		Enterobacter cloacae 811101	Glucose	Batch	0.20	7.0	37	1.69	2.9[j]	
Fe	5.0	Anaerobic sludge	Glucose	Batch	0.08	5.5	37	338[d]	37.0	[62]
	100	*Enterobacter cloacae* DH-89	Glucose	Batch	0.10	7.0	37	1.90	100	[58]
	400	Mixed bacterial consortium	Glucose	Batch	0.25	7.0	30	1.23	38.0	[61]
	250	Mixed culture and *Clostridium butyricum* TISTR	Water hyacinth	Batch	0.08	7.0	35	57.0[d]	55.0	[60]
	312	*Rhodobacter sphaeroides* NMBL-02 + *Escherichia coli* NMBL-04	Malate	Batch	0.06	5.6	32	3.10[e]	19.4	[55]
Fe_2O_3	175	*C. acetobutylicum* NCIM2337	Glucose	Batch	0.20	6.0	37	2.33	33.9	[57]
	800	*Clostridium pasteurianum* CH5	Glucose	Batch	0.10	7.0	35	2.20	10.0	[27]
	200	*Enterobacter aerogenes* ATCC13408	Glucose	Batch	0.20	6.0	37	1.55	17.0	[63]
	125	*E. cloacae* 811101	Glucose	Batch	0.20	7.0	37	2.07	21.8	[26]
	50.0	Anaerobic sludge	Glucose	Batch	0.20	5.5	60	1.92	53.6	[20]
	200	*E. cloacae* 811101	Sucrose	Batch	0.20	7.0	37	2.72	4.8	[26]
	200	Mixed culture	Sucrose	Batch	0.80	6.0	35	1.78	33.0	[64]
	200	*E. aerogenes* ATCC13408	Cassava starch	Batch	0.20	6.0	37	124[d]	63.1	[63]
	50.0	Anaerobic sludge	Dairy wastewater	Batch	0.10	5.5	37	16.75[f]	24.0	[45]
	200	Anaerobic sludge	Molasses wastewater	Batch	0.10	5.5	37	7.85[f]	44.0	[46]
	25.0 mg/g VSS	Anaerobic sludge	Starch wastewater	ABR	30.0	6.7	30	0.90	57.8	[65]
Fe_3O_4	400	Anaerobic sludge	Glucose	Batch	0.08	7.0	35	1.53	26.4	[66]
	50.0	Mixed culture	Wastewater	Batch	0.10	6.0	37	44.3[d]	83.3	[56]
	200	Anaerobic sludge	Sugarcane bagasse	Batch	0.10	5.0	30	1.21	69.6	[67]
Ni	2.5	Anaerobic sludge	Glucose	Batch	0.08	5.5	37	250[d]	0.90	[62]
	5.7	Anaerobic sludge	Glucose	Batch	0.70	5.6	33	2.54	22.7	[68]
	60	Anaerobic sludge	Watewater	Batch	0.25	7.0	55	24.7[d]	23.0	[19]
NiO	200	Anaerobic sludge	Glucose	Batch	0.20	5.5	60	1.30	4.8	[20]
	10.0	Anaerobic sludge	Dairy wastewater	Batch	0.10	5.5	37	15.7[f]	16	[45]
	5.0	Anaerobic sludge	Molasses wastewater	Batch	0.10	5.5	37	6.73[f]	23.5	[46]
Pd	5.0	*E. cloacae* 811101	Glucose	Batch	0.20	7.0	37	1.48	0.6	[29]
	5.0	Mixed culture	Glucose	Batch	0.20	7.0	37	2.48	6.4	
SiO_2	40.0	*Chlamydomonas reinhardtii* CC124	Air:CO_2 (97:3)	PBR	110	4.2	28	0.61[g]	45.2	[70]
	5.1	*C. butyricum* CWBI1009	Glucose	Batch	0.20	7.6	30	0.96	4.3	[44]
	120	Acidogenic mixed culture	Wastewater	Cont.	0.16	5.5	28	7.02[h]	666	[48]

Table 1 continued

Nano-particles		Organisms	Feed	Process parameters				Yield[c]	H_2 yield efficiency (%)	Refereces
Composition	Conc. (mg/l)[a]			Mode	WV (L)	pH	Temp. (°C)			
TiO_2	50.0	*C. pasteurianum* CH5	Glucose	Batch	0.10	7.0	35	2.10	5.0	[27]
	100	*Rhodopseudomonas palustris*	Waste sludge	Batch	0.30	8.0	30	1.01[f]	46.1	[50]
	60.0	*R. sphaeroides* NMBL-02	Malate	Batch	0.10	8.0	32	1.75[g]	69.9	[71]
Inorganic mixtures										
Fe + Ni	37.5 + 37.5	Anaerobic sludge	Starch	Batch	0.80	7.0	37	150[d]	200	[59]
Fe_2O_3 + NiO	200 + 5.0	Anaerobic sludge	Molasses wastewater	Batch	0.10	5.5	37	8.83[f]	62.0	[46]
	50.0 + 10.0	Anaerobic sludge	Dairy wastewater	Batch	0.10	5.5	37	17.2[f]	27.0	[45]
Organic										
Granular activated carbon	100	Anaerobic sludge	Glucose	UASB	5.00	6.5	25	1.42	na	[47]
	10,000	Acidogenic mixed culture	Starch wastewater	Cont.	0.16	5.5	28	2.12[h]	94.5	[48]
Powdered activated carbon	33.0	Anaerobic sludge	Sucrose	Batch	0.06	5.5	37	1.30	62.5	[75]
	33.3	Anaerobic sludge	Sucrose	UASB	0.06	5.5	37	1.30	73.0	[74]
	5000	Acidogenic mixed culture	Starch wastewater	Cont.	0.16	5.5	28	1.57[h]	44.0	[48]
Carbon nanotubes	100	Anaerobic sludge	Glucose	UASB	5.00	6.5	25	2.45	na	[47]

EAB electrochemically active biofilm reactor, *ABR* anaerobic baffled reactor, *PBR* photobioreactor, *UASB* upflow anaerobic sludge blanket reactor

[a]Optimum concentration

[b]Concentration in nM

[c]mol/mol of hexose

[d]L/kg TS or COD or VS

[e]mol/mol of substrate

[f]mol/kg COD

[g]Average production rate in ml H_2/l/h

[h]mol/kg COD-day

[i]Not available

[j]Negative effect

maximum production of 1.48 and 2.48 mol H_2/mol glucose was recorded as compared to their respective controls. Here, the lag phase H_2 production was also reduced in the presence of PdNPs. On the contrary, the Pd^{2+} ions negatively influenced yields and lag phase of H_2 production under similar conditions. Overall, mixed culture showed higher H_2 yield than *E. cloacae* 811101. Remarkably, the supplementation of PdNPs up to 20.0 mg/l did not affect the bioactivity of both *E. cloacae* and mixed culture. Here, Pd^{+2} ions showed high inhibitory effect on H_2 production as compared to PdNPs, which was evident with significant reduction in the glucose conversion efficiency under similar conditions. Further, higher production of propionate as intermediate metabolites confirmed its negative effect of H_2 production [29]. The higher hydrogenase activity in the presence of PdNPs might be directly linked to high H_2 production over Pd^{2+} ions [29, 54].

Iron and Iron Oxide

Many bacterial cultures have been evaluated for H_2 production in the presence of Fe NPs (FeNPs) using sugars and biowaste materials as a feed [55–61]. Taherdanak et al. [62] have demonstrated the effects of FeNPs versus Fe^{2+} ions in the concentration ranges of 0–50 mg/l on fermentative H_2 production by anaerobic sludge from glucose. Both, Fe^{2+} ions and FeNPs showed an enhancement in H_2 yield of 15.0 and 37.0% at concentrations of 10 and 25 mg/l compared to that recorded with controls (247 l/kg VS). Here, high H_2 production was associated with major shift

in the intermediate metabolites towards higher acetate to butyrate ratio and decrease in the ethanol and propionate concentrations. Interestingly, FeNPs showed 75% reduced propionate production as compared with 35% in presence of Fe^{2+} ions [62]. On the other hand, Nath et al. [58] showed the influence of green synthesized FeNPs using bark and leaf extracts of *Syzygium cumini* and Fe^{2+} ions at higher concentration of up to 200 mg/l on H_2 production by *E. cloacae* DH-89. A similar positive effect on the H_2 yield was observed in the presence of FeNPs over Fe^{2+}ions. FeNPs (100 mg/l) exhibited enhancement of 100% (1.9 mol H_2/mol hexose) than the control (0.95 mol H_2/mol glucose). In contrast, a maximum H_2 yield of 1.45 mol/mol glucose was recorded in the presence of 25 mg/l of Fe^{2+} ions. Interestingly, FeNPs resulted in higher cell growth of *E. cloacae* DH-89. Thus, these results suggested that FeNPs enhance the metabolic process of *E. cloacae* DH-89 for H_2 production [58]. On the other hand, FeNPs at a high concentration of 400 mg/l showed lower enhancement of 38% in H_2 yield (1.23 mol/mol hexose) by a mixed bacterial consortium from glucose as feed [61]. Here, the higher Shannon diversity index of 4.527 as compared with control (4.173) suggested that microbial diversity was more stable in presence of FeNPs. It was confirmed with higher abundance of 60.8% of the dominant strain of *Clostridium* sensu *sticto* in the microbial consortium over 51% in control conditions. Mixed culture composed of sludge from sewage water, anaerobic digested sludge, cow dung, soil from wheat field, and lake sediment saturated with *C. butyricum* TISTR exhibited H_2 production of 57 l/kg TS at a concentration of 250 mg/l of FeNPs from water hyacinth [60]. In this case, an enhancement of 55% in the H_2 yield was observed. Remarkably, no harmful effect of NPs on H_2 production was reported at high concentration of 500 mg/l [60]. Dolly et al. [55] have demonstrated the photo-fermentative H_2 production by co-culture of *E. coli* NMBL-04 and *Rhodobacter sphaeroides* NMBL-02 from malate in the presence bulk- and NPs forms of Fe at broad ranges of concentration i.e., 1–700 mg/l. The nano form of Fe particles were found to be 19.4% more efficient in yielding H_2 (3.1 mol/mol substrate) than the bulk-form at optimum concentration of 312 mg/l.

Pure cultures, including *C. acetobutylicum* NCIM2337, *E. aerogenes* ATCC13408 and *E. cloacae* 811101 using glucose as feed showed an enhancement in H_2 production yield of 33.9, 17.0 and 21.8% at a concentration of 175, 200 and 125 mg/l of Fe_2O_3 NPs, respectively [26, 57, 63]. Among these organisms, *C. acetobutylicum* NCIM2337 exhibited maximum yield of 2.33 mol H_2/mol glucose [26]. Interestingly, Mohanraj et al. [26] has suggested that the type of sugar as feed also significantly influence the H_2 production in the presence of Fe_2O_3 NPs (200 mg/l) by *E. cloacae* 811101. Glucose was founded a more ideal feed to achieve 21.8% higher H_2 yield over sucrose (4.8%). On the other hand, mixed culture performed efficiently by enhancing 33% yield (1.78 mol H_2/mol hexose) from sucrose at the same concentration of Fe_2O_3 NPs [64]. Similarly, Gadhe et al. [45, 46] have suggested that wastewaters as feed from diverse sources also required different concentrations of Fe_2O_3 NPs for optimum H_2 production by anaerobic sludge. Dairy wastewater showed maximum H_2 production of 16.75 mol/kg COD at 50 mg/l of Fe_2O_3 NPs [45]. Whereas, molasses wastewater exhibited lower production of 7.85 mol H_2/kg COD at much higher concentration of 200 mg/l [46]. Overall, enhancement of 24 and 44% was observed from dairy and molasses wastewaters as feed, respectively. Nasr et al. [65] showed that immobilization of anaerobic sludge on Fe_2O_3 NPs is beneficial to improve yield by 57.8% (0.90 mol H_2/mol glucose) from starch as feed. Further, it was also suggested that integration of dark- to photo-fermentative process may prove effective to achieve higher H_2 yield [65]. On the other hand, *E. aerogenes* ATCC13408 exhibited quite higher enhancement of 63.1% in H_2 yield (124 l/kg TS) from cassava starch at 200 mg/l of Fe_2O_3 NPs [63], which was nearly fourfold better than that recorded with pure glucose as feed by *E. aerogenes* ATCC13408 (17.0%). Brief morphological analysis of *E. aerogenes* ATCC13408 suggested that the higher cell aggregation was observed at 200 mg/l of Fe_2O_3 NPs. This phenomenon was likely to be related with response to NPs as the formation of bacterial nanowire, as it played an important role in enhancement of electron transfer among the cells during the fermentation. This was confirmed by the cellular internalization of Fe_2O_3 NPs as dark spots (30 nm) in cytoplasm of *E. aerogenes* ATCC13408 [63]. Zao et al. [66] reported an enhancement of 26.4% in the production yield (1.53 mol H_2/mol glucose) by anaerobic sludge at higher concentration of 400 mg/l of Fe_3O_4 NPs under mesophilic conditions. Similarly, anaerobic sludge showed 53.6% improvement in H_2 yield (1.92 mol/mol hexose) under thermophilic conditions at 60 °C [20]. In contrast, distillery wastewater was found to be a suitable feed to achieve an enhancement of 83.3% in H_2 yield (44.3 l/kg COD) in the presence of lower concentration of 50 mg/l of Fe_3O_4 NPs by mixed culture [56]. Sugarcane bagasse using anaerobic sludge as inoculum showed enhancement of 69.6% in the yield (1.21 mol H_2/mol hexose) by 200 mg/l supplementation of Fe_3O_4 NPs, which was higher than the Fe^{2+} ions (62.1%) [67]. Microbial community structure and hydrogenase gene expression analysis suggested that Fe_3O_4 NPs exhibited highest concentration of H_2 producing communities and hydrogenase gene as compared with both control and in presence of Fe^{2+} ions. Further, an increase the concentration of Fe_3O_4 NPs to 400 mg/l exhibited significant adverse

effect on yield (0.62 mol of H_2/mol hexose). Here, high concentration of NPs may lead to toxicity and generation of reactive oxygen species that results a negative influence the growth of microorganism [67].

Nickel and Nickel Oxide

Ni^{2+} ions have been well known to improve the H_2 production yield though the enhancement in catalytic activity of hydrogenases enzyme [59, 62]. Taherdanak et al. [62] has demonstrated the influence of both Ni^{2+} ions and Ni NPs (NiNPs) on H_2 production by anaerobic sludge from glucose as feed. NiNPs up to 2.5 mg/l showed insignificant results as 0.9% higher H_2 production yield than the control (247 l/kg VS), which was drastically decreased to 99 l/kg VS at high concentration 50 mg/l of NiNPs [62], whereas, Ni^{2+} ions exhibited significant higher enhancement in the H_2 yield by 55.0% at the concentration of 25 mg/l. Overall, the supplementation of NiNPs were not favorable for H_2 production by anaerobic sludge [62]. In contrast, Mullai et al. [68] showed a 22.7% enhancement with maximum H_2 production yield of 2.54 mol/mol glucose by anaerobic sludge in the presence of 5.7 mg/l of NiNPs. Also, anaerobic sludge exhibited similar improvement in H_2 yield of 22.7% from the wastewater as feed under the thermophilic condition (55 °C) [19]. Similarly, anaerobic sludges showed a quite variable enhancement in the H_2 production with industrial wastewater sources from dairy and molasses using NiO as a NPs [45, 46]. Molasses showed an enhancement of 23.5% over dairy wastewater (16%) at the NiO NPs concentration of 10 and 5 mg/l, respectively. On the other hand, glucose resulted in lesser improvement in the yield of 4.8% (1.30 mol H_2/mol hexose) at significantly higher concentration 200 mg/l of NiO NPs [20]. Here, the variation in the H_2 production yields might be associated with the differences in the composition of the feed.

Silica and Titanium Oxide

Silica has been well recognized as more biocompatible support towards the both proteins and microorganisms [44, 69, 70]. Venkta Mohan et al. [48] demonstrated effectively use of mesoporous SiO_2 particles in H_2 production using mixed consortia from the chemical wastewater of common effluent treatment plant. Generally, high feed loading resulted in adverse effect on the H_2 production by mixed consortia due to low degradation or inefficient utilization of feed [48]. Remarkably, SBA-15 silica particles (120 mg/l) stimulated significant higher H_2 production up to 666% (7.02 mol/kg COD-day) as compared with control at high loading of feed (2.55 kg COD/l-day) through self-immobilization of cells on the particle during the fermentation conditions [48]. This high H_2 yield was associated with an efficient feed degradation of 37.6% as compared with control (23.1%). Further, high and low level of acetate and propionate, respectively as a soluble metabolites confirmed the enhancement in H_2 yield in the presence of SBA-15 than the control. Beckers et al. [44] showed that porous SiO_2 particles (5.1 mg/l) did not significantly influence the H_2 production yield and the metabolite intermediate profile of *C. butyricum* CWBI1009 from glucose as feed. Here, only 4.3% increase in the H_2 yield (0.96 mol/mol glucose) was observed. On the other hand, microalga *Chlamydomonas reinhardtii* CC124 in the presence of SiO_2 particles (60 mg/l) exhibited 45.2% higher H_2 production and an average rate 0.61 ml/l/h as compared with control experiment under photo-fermentative conditions [70]. Interestingly, the growth of *C. reinhardtii* CC124 was positively influenced in presence SiO_2 particles with 23% net increases in chlorophyll concentration during H_2 production associated with improved light distribution.

Zhao and Chen [50] evaluated the effect of TiO_2 NPs (25 nm) on the growth, activities of H_2 production enzymes (nitrogenase and H_2-uptake) for photo-fermentative process by *R. palustris* from dark-fermentative effluent. In the presence of TiO_2 (100 mg/l), the H_2 production and nitrogenase activity were significantly enhanced over the control. In contrast, up-take hydrogenase activity decreased significantly in the presence of TiO_2. Therefore, higher H_2 production was achieved as low consumption of produced H_2 by up-take hydrogenase. Biomass of *R. palustris* also increased. An enhancement of 46.1% in the H_2 production was observed in presence of TiO_2 NPs with the yield of 1.01 mol/kg TS. Overall, the two stage H_2 production with total yield of 1.88 mol/kg TS suggested that integrative approach of dark- followed with photo-fermentative approach is suitable for achieving high H_2 recovery efficiency from waste sludge [50]. Similarly, *R. sphaeroides* NMBL-02 showed enhancement of 1.7- and 1.9-fold in the average H_2 production rate and duration as compared with control using 60 mg/l of TiO2 NPs under the photo-fermentative conditions [71]. Indirectly, Jafari and Zilouei [72] suggested that TiO_2 pre-treated biomass showed significant H_2 production of about 101 l/kg VS as compared to control (44.2 l/kg VS). Similarly, encapsulation of *C. reinhardtii* L159I-N230Y cells with TiO_2 shells was found to lead to about two fold higher efficiency for H_2 production (100 ml/l) than the free living cells [73].

Mixture of NPs

The individual influence of Fe and Ni metals has been widely reported for their positive effects on activity of the hydrogenases [45, 46, 62]. Therefore, the combined influence of Fe and Ni NPs was evaluated to improve the H_2

production by anaerobic using the different concentrations (0–50 mg/l) of Fe and Ni NPs mixture sludge from starch as feed [59]. The maximum H_2 production of 150 l/kg VS was observed at Fe and Ni concentration of 37.5 and 37.5 mg/l, respectively. Here, an enhancement of nearly 200% in H_2 yield was observed as compared to controls. Interestingly, individual NPs (25 mg/l) showed maximum H_2 production of 66.8 and 61 l/kg VS for Fe and Ni, respectively. Similarly, combined effect NiO and Fe_2O_3 NPs on H_2 production by anaerobic sludge was evaluated from molasses and dairy wastewater [45, 46]. From dairy wastewater, the maximum H_2 production of 17.2 mol/kg COD was observed in the presence of Fe_2O_3 (50 mg/l) and NiO (10 mg/l), respectively. About 27% enhancement in the H_2 yield was shown by co-addition of NPs as compared with controls. Remarkably, the optimum addition of these NPs resulted in a significant decrease in lag phase of H_2 production from 3.6 to 2.8 h [45]. This enhancement in H_2 production associated with enhancement of the ferredoxin, hydrogenase and ferredoxin oxidoreductase enzymes, by the surface area and quantum size of NPs. On the other hand, maximum H_2 production was observed to be 8.83 mol/kg COD using Fe_2O_3 (200 mg/l) and NiO (5 mg/l) NPs from complex distillery wastewater [46]. Here, an enhancement of 62% in H_2 yield was observed as compared with control. Interestingly, the H_2 production rate was enhanced by 221% in the presence of co-addition of NPs.

Organic Particles

CNTs are a unique tubular structural material of carbon. This has known applications in the biosensors and microbial fuel cells due to its effective role in the reduction of potential for the redox reactions and electron transfer kinetics [47]. The UASB reactor using CNTs (100 mg/l) showed efficient production of 2.45 mol H_2/mol glucose at HRT of 24 h by anaerobic sludge as inocula [47]. Under similar conditions, anaerobic sludge without CNTs was washed out of the reactor within two weeks of operation. As compared with granular activated carbon (1.42 mol H_2/mol glucose), CNTs based UASB reactor showed 1.7-fold higher yield of H_2. Similarly, activated carbons in the granular (10 g/l) and powder (5 g/l) forms showed the significant improvement in the H_2 production i.e., 94.5 and 44.0% using acidogenic mixed culture from the starch waste as a feed, respectively [48]. Activated carbon in the concentrations of 33.0 and 33.3 mg/l were found very effective in enhancing H_2 production yield by 62.5 and 730% under batch and UASB mode from sucrose using anaerobic sludge as inocula, respectively [74, 75].

Opinion

The lower H_2 production yield by organism to the theoretical values of 4 mol/mol of hexose and high cost of the feed are the major limiting factors for the large scale production under dark-fermentative conditions [9, 31]. Therefore, to enhance the H_2 production, various strategies has been adapted to overcome these problems, including optimization of process parameters, screening of potential H_2 producers and use of low cost feed such as biowaste substrate [15, 16, 34]. The hydrogenases are key enzymes involved in the BHP and their activity was significantly influenced with the Fe^{2+} and Ni^{2+} metal ions. Recently, the NPs forms of these metals along with others including, activated carbon, Ag, Au, CNTs, Pd, Si and Ti also resulted in the profound effect of up to 6.7-fold on the H_2 production yield [27, 29, 45, 48, 49, 51, 55, 58, 62]. Here, the enhancement in the H_2 production is associated with NPs concentration and their properties. In the contrast, CuNPs exhibited the negative effect on the H_2 production yield at lower concentration of 2.5 mg/l [30]. The variable influences of NPs or their mixtures on the BHP was observed using pure and mixed culture from both sugars and biowastes as a feed. These NPs mostly enhance the H_2 production yield through their significant positive effects on the organism growth, feed degradation efficiency and intermediate metabolites profile. Primarily, in the presence of NPs H_2 producers shift intermediate metabolites towards higher ratio of acetate to butyrate and inhibition in the production of ethanol and propionate production. Among, the pure cultures, including *C. butyricum* CWBI1009, *C. pasteurianum* CH5, *E. aerogenes* ATCC13408, *E. cloacae* DH-89, *E. cloacae* and 811101 and *R. palustris* strains, *E. cloacae* DH-89 exhibited the maximum enhancement in H_2 yield up to 100% [58]. On the other hand, the mixture of Fe and Ni NPs were found to be more suitable to improve H_2 yield up to 200% using anaerobic sludge as a mixed culture [59]. Similarly, undefined mixed consortia showed maximum increase in the H_2 yield up to 666% using SiO_2 particles [48]. Most of the times, synthesized NPs with physical and chemical method were used in the BHP. Due to high biocompatibility of biologically synthesized NPs over synthesized through physical and chemical methods, more research is needed in this area for their potential application in BHP [58]. The microbial community comparison of anaerobic sludge for the H_2 production in the presence of NPs suggested that microbial structure was significantly changed [61]. Thus, this approach can be adapted to improve the selective H_2 producers enrichment during the fermentative process. Also, NPs were effective during pre-treatment of biomass and immobilization of whole cells, which were used for the H_2

production, implying their high potential applications [48, 72, 73, 76]. Further, the integration of dark-fermentative H_2 production with other process such as photo-fermentative H_2 production, CH_4 production or polyhydroxyalkanoates have been suggested as more effective approaches in multiple stage system for improving the process economy [3, 7, 39, 40, 50, 77–80]. Therefore, more integrative processes are needed to evaluate the role of NPs. These processes can be effectively used to develop multi-stage system using CH_4 or simulated biogas to produce more biofuels such as methanol or value added bio-products using methanotrophic organism for the sustainable development [81–83].

Acknowledgements The authors wish to thank the Director of CSIR–Institute of Genomics and Integrative Biology, Delhi, India, CSIR Project WUM (ESC0108) for providing the necessary funds, facilities and moral support. This research was also supported by KU Research Professor program of Konkuk University.

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