



A CONTEMPORARY IMPROVEMENT IN FERMENTATIVE BIO HYDROGEN PRODUCTION - REVIEW

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Abstract:

Hydrogen verve, as a kind of clean energy with great latent, has been a hotspot for study worldwide. Based on the recent research on biohydrogen production, this paper gives a brief analysis on the following aspects: fermentative hydrogen production process and the engineering control statagy, key factors affecting the efficiency of hydrogen production, such as substrates, cysteine, metal ions, anaerobic fermentation terminal products, and formic acid and ammonia. Additionally, anaerobic fermentative hydrogen-producing strain and regulation and control of enzyme gene in fermentative hydrogen production are also discussed. Finally, the prospect of anaerobic fermentative biohydrogen production is proposed in three study areas, namely developing new techniques for breeding hydrogen-producing bacteria, exploitations of more strains and gene resources, and intensifying the application of microbial molecular breeding in hydrogen production.

1. Introduction:

Fossil energy source cannot be rejuvenated and will be pooped with increasingly more fossil fuel depletions. Of other alternative energy sources with potential, hydrogen energy is a kind of new energy source with abundant reserves, not depending on fossil fuel. Moreover, hydrogen energy conforms to the requirement of worldwide environmental protection, thus has received more attention all over the world. Hydrogen may be produced in biosystem, which includes two ways of light-drive process and anaerobic fermentation, the former is theoretically perfect process with transforming solar energy into hydrogen by photosynthetic bacteria directly. However, due to the low utilization efficiency of light and difficulties in designing light reactor, this method is hard to be applied in practice. The latter carries out anaerobic fermentation by the hydrogenogens, which has many advantages, such as rapid, simple, easy operation, and hydrogen production by renewable resources and organic waste [1]. Compared with the light-drive reactor, anaerobic fermentative hydrogen-production is easier to conduct and suitable for the demands of sustainable development strategy. However, the yield and rate of hydrogen production are still low at present. With the rapid development of molecular biological technology, the directional heredity reconstruction for microbe has been a new research hotspot, which can radically change microbial biological properties and metabolic modes to cultivate superior microbial strains more beneficial to biohydrogen production, economize costs and increase production efficiency and yield, and provide more efficient pathways for the exploitation and popularization of hydrogen energy sources.

2. Fermentative Hydrogen Production Process and the Engineering Control Strategy:

2.1. Process of Hydrogen Production:

The hydrogen-producing fermentative type in biological reactor determines the hydrogen-producing efficiency. The acid-producing fermentation of organic waste water has three types: butyric acid-type fermentation, metacetic acid-type fermentation, and ethanol-type fermentation. In parallel, there are three acid-producing fermentation floras: butyric acid-type fermentation flora, metacetic acid-type fermentation flora, and ethanol-type fermentation flora. The ethanol-type fermentation flora has a higher gas-producing speed, a higher hydrogen-producing speed, a greater ratio of hydrogen-producing speed and a higher level of hydrogen content than metacetic acid-type fermentation flora [2].

Li et al. [3] reviewed the technological design and flow on biohydrogen production system, and pointed out that we should pay more attention to reaction substrate, sludge inoculation and quick starting, engineering monitoring, and production inhibition in the technological flow. Biohydrogen production could be conducted by the methods of activated sludge processes and immobilized cells. Moreover, Li et al. also discussed the effects of ecological factors and parameters of engineering control on hydrogen production, such as temperature, pH, oxidation–reduction potential, hydraulic retention time, velocity, power and alkalinity of mixer.

Li et al. [4] established the predominant flora and optimal parameters of engineering control for an ethanol-type fermentation process. Zhang et al. [5] produced hydrogen by *Clostridium* fermentation in an unsaturation flow reactor, and they designed a kind of mesophilic unsaturation flow reactor to determine the hydrogen yield in glucose fermentation. At the same time, some researchers produced hydrogen by high-efficient carrier-induced granular sludge bed bioreactors [6] and synthesis models of ferronickel hydrogenase [7].

Zhang et al. [8] successfully achieved anaerobic biological hydrogen productions in two lab-scale anaerobic hydrogen production reactors under mesophilic (37 °C) and thermophilic (55 °C) conditions, respectively. The mesophilic reactor was operated over 4 months by seeding with river sediments and feeding with glucose solution, in which the highest hydrogen production rate was 8.6 (L/(L d)) and the substrate hydrogen production molar ratio (H₂/glucose) was 1.98.

2.2. Key Factors Affecting the Efficiency of Hydrogen Production:

2.2.1. Substrates:

The hydrogen-producing bacteria are quite sensitive to pH fluctuation because pH change may result in the change of their metabolic pathway. However, the bacteria for pH change have strong balancing and regulating abilities. The optimal pH for ethanol-type fermentative bacteria ranges from 4.0 to 4.5. Moreover, the hydrogen-producing speed of biohydrogen reactor rapidly increases with the increase of organic load, with the optimal organic load of 40–55 kg COD/m³ d [9]. Zuo et al. [10] used pre-heated river sediments as seed sludge to conduct anaerobic bio-hydrogen production. A series of batch experiments were performed to investigate the effects of several factors on anaerobic bio-hydrogen producing process. The results showed that several factors, such as substrate and its concentration, temperature, and initial pH, could also affect the anaerobic bio-hydrogen production at different levels. At the organic loading rate of 36 kg COD/m³ d, the highest hydrogen production was 6.3–6.7 L H₂/L reactor d, the specific hydrogen production was 1.3–1.4 mol H₂/mol glucose, and the hydrogen content in the gas was 52.3%.

Kim et al. [11] compared the performances of a continuous-flow stirred-tank reactor (CSTR) and an anaerobic sequencing batch reactor (ASBR) for fermentative hydrogen production at various substrate concentrations. Result showed that hydrogen production was dependent on substrate sucrose concentrations, resulting in the highest performance at sucrose concentration of 30 g COD/L. At the lower sucrose concentrations, the hydrogen yield decreased with biomass reduction and changes in fermentation products. Lin et al. [12] studied the cooperation of hydrogen-producing fermentation bacteria (HPFB) in mixed culture at a batch test, and result showed that the cooperation of mixed cultured bacteria was conditional on the substrates. When fed with glucose which is easily utilized by hydrogen-producing bacteria, the hydrogen-producing ability of hydrogen-producing bacteria was restrained because of the competition for the co-substrate between hydrogen-producing bacteria and other fermentation bacteria, and it was quite difficult for the cooperation of mixed culturing bacteria to be performed. When fed with complex organic substance, the hydrogen-producing ability of hydrogen-producing bacteria was enhanced via the cooperation of mixed cultured bacteria. Furthermore, the combination of alkali pretreatment with high initial pH not only promoted the growth of main hydrogen-producing anaerobe, but also restrained the hydrogen-consuming anaerobe. Some researchers, based on clostridia as predominant flora and cellulose as materials, conducted hydrogen production by activated sludge mesophilic anaerobic fermentation. The results showed that high hydrogen generation from cellulose was associated with low ratio of initial cellulose concentration to initial sludge density (S_0/X_0) [13].

2.2.2. Cysteine:

During the pure cultivation of anaerobic bacteria, as a reducer, cysteine is added into culture medium to decrease redox electric potential and bring the system under the complete anaerobic conditions. However, for B49 (an anaerobic bacterium strain), cysteine also has the function similar to growth factor. Cysteine has important status and action for the structure and function of Fe-S protein, which may be the main factor promoting B49 hydrogen production [9].

Lin et al. [14] proposed LM series cultures, and determined that the reducer was l-cysteine and the optimal pH was 6. Five isolated strains of high hydrogen-producing fermentative bacteria were identified, which belong to 4 genera. Among these genera, Bacteroides and Klebsiella do not belong to the familiar genus of isolated high hydrogen-producing fermentation bacteria in the world. The isolation of high effective hydrogen-producing bacteria by LM-1 culture was highly effective.

2.2.3. Metal Ions:

At the cellular level, some metal ions have certain effects on the activity and number of hydrogen-producing bacteria. For example, Fe shortage could influence the growth, metabolism, and hydrogen-producing ability of B49. This suggests that adding Fe^{2+} may increase the specific activities of hydrogen enzyme and NADH-Fd reductase of hydrogen-producing fermentation bacteria, and consequently enhance its hydrogen-producing ability [9]. Wang et al. [15] found that the bacterium fermentation type could turn into ethanol-type fermentation from butyric acid-type fermentation by adding Fe. In bacterial metabolism process, pure Fe could increase the abilities of fermentation and hydrogen-producing of bacteria. Furthermore, Mg^{2+} is also an important influencing factor. During the process of glycolysis, about 10 enzymes in cytoplasm need to be activated by Mg^{2+} . Mg^{2+} shortage may limit the growth anabolism of hydrogen-producing fermentative bacteria (such as B49), and its hydrogen-producing ability.

Thus, adding Mg²⁺ can promote the growth of ethanol type hydrogen-producing fermentative bacteria and enhance its hydrogen-producing ability.

2.2.4. Anaerobic Fermentation Fatal Products:

Anaerobic fermentation fatal products can affect hydrogen-producing ability and metabolic process of fermentation microflora. For ethanol fermentation, high ethanol production is simultaneously achieved with high hydrogen production under the equal quantities of aqueous terminal products. Our hydrogen-producing experiments of ethanol and acetic acid additions convinced that ethanol had little inhibitory effect on fermentative hydrogen production, and acetic acid had strong inhibitory effect on hydrogen production [16].

Van Ginkel and Logan [17] studied the inhibition of biohydrogen production by using undissociated acetic and butyric acids. Glucose fermentation to hydrogen resulted in the production of acetic and butyric acids. Hydrogen yields were inhibited more by self-produced acids (produced at high glucose feed concentrations) than by similar concentrations of externally added acids (lower glucose feed concentrations).

2.2.5. Formic Acid and Ammonia:

When 20 mM formate was separately added to pH 6.3 and pH 5.8 *Enterobacter aerogenes* glucose cultures (formate culture) at the beginning of cultivation, hydrogen evolution through both glucose consumption and decomposition of the extrinsic formate occurred together, while hydrogen evolution occurred only through glucose consumption in the control cultures. The decomposition rate of the extrinsic formate in the pH 5.8 formate culture was faster than that in the pH 6.3 formate culture. The hydrogen yield from glucose in the pH 6.3 formate culture increased due to the increasing amount of the nicotinamide adenine dinucleotide for hydrogen production [18]. Salerno et al. [19] investigated the inhibition of ammonia for biohydrogen production in batch and continuous flow reacts with glucose as a substrate. They concluded that the hydrogen production could be possibly made at high concentrations (up to 7.8 g N/L) of ammonia in continuous flow systems as long as the reactor is initially acclimated to a lower ammonia concentration (<0.8 g N/L).

3. Anaerobic Fermentative Hydrogen-Producing Strain:

Isolation and identification of high efficient hydrogen-producing bacteria not only laid the basis of resolving the industrialization of hydrogen production by fermentation, but also provided important microbial species resources for the research on genetic improvement and physiology and biochemistry of hydrogen-producing bacteria [20]. Despite the ratio of hydrogen production of majority of fermentation hydrogen-producing strains being higher than those of photosynthetic microorganism [4] and [21], highly efficient hydrogen-producing strains still need to be screened further to enhance hydrogen-producing ability.

Anaerobic fermentative microbial species may be obtained from natural environment or artificial isolation and screening in laboratory [22]. Iyer et al. [23] investigated the characteristics of hydrogen-producing bacterial communities from a heat-treated soil inoculum. Ribosomal intergenic spacer analysis fingerprints at 37 °C from the 10 h HRT bioreactor exhibited a clear shift from populations related to *Clostridium acidisoli* (subclusterIc) at 30 °C to populations related to *Clostridium acetobutylicum* (subclusterIb).

Li et al. [24] analyzed the effect of different carbon/nitrogen (C/N) ratios on hydrogen production and alcohol dehydrogenase (ADH) activities in the acidophilic strain X-29 using a batch test. The results indicated that strain X-29 grew well and had strong hydrogen production under low pH. Others [25] isolated a highly efficient

biohydrogen production bacterium by anaerobic digesting of organic waste water, the strain was different from Clostridium species with a temporary nomenclature of genus of Biohydrogen bacterium Gen.Nov.Sp.Nov., and it could produce hydrogen by molasses waste water. Li et al. [26] later isolated more than 90 strains of hydrogen-producing bacteria by various anaerobic culture techniques, of those, Rennanqilyf 3 was studied further. Through 16S rDNA sequencing and comparison verification, this strain was a novel species of bacterium not reported at present, and its gas-producing amount and growth state were determined under the condition of an intermittent experiment. The strain Rennanqilyf 3 possessed the maximal cell growth amount of 0.46 at 15.0 g/L of glucose and maximal gas-producing amount of 58.6 mmol/L at 12.0 g/L of glucose; and its optimal pH of growth and hydrogen-producing amount was about 5.5. Moreover, mutagenesis techniques were often utilized in microbe breeding [27].

Ren et al. [2] found a new genus of fermentative hydrogen-producing bacteria, including the strains Rennanqilyf 1 and B49. Of these, the strain B49 has a good acid resistance and a higher hydrogen production, and it is beneficial to engineering applications. Under the condition of stirring cultivation, the optimal pH of fermentative hydrogen production and bacteria growth for B49 was basically identical, ranging from 3.9 to 4.2 [2] and [28]. The accession number of B49 in EMBL was AF481148 [14], and B49 had been determined as a strain of new genus – Ethanoligenens by the analysis of physiological characteristics and 16SrDNA sequence. Hydrogen-producing behavior of Ethanoligenens sp. B49 did not depend on iron. This characteristic is similar to that of Clostridium butyricum but quite different from that of Enterobacter cloaca with Coli-type hydrogen-producing pathway. Therefore, the hydrogen production by Ethanoligenens sp. B49 is probably by Clostridial type pathway [29]. Lactobacillus paracasei also has the ability of hydrogen production, and continuous hydrogen production from municipal organic waste is enabled [30].

Xing et al. [31] isolated two special anaerobic hydrogen-producing bacterial strains of YUAN-3T and X-29 in continuously stirred reactor, and both of them are mesophilic Gram-positive bacteria without generating spore. On the basis of the distant phylogenetic relationship with related taxa, unique chemotaxonomic characteristics, DNA G+C content and physiological and biochemical traits, it is evident that YUAN-3T and X-29 represent a distinct genus within the Clostridium leptumrRNA subgroup. Its taxonomic name is Fthanoligenerasharhiraense Gen. Nov.Sp.Nov. Only one species, Fthannligenensharhinense, has been described so far, and this species has been designated the type species. The type strain is YUAN-3T, which formed large auto-aggregative granule on shake culture. Specific hydrogen conversion rate and hydrogen production rate of strain YUAN-3T were 2.81 mol H₂/mol-glucose and 27.6 mmol H₂/g-dry cell h, respectively, and this strain has been the only auto-aggregative in reported hydrogen-producing bacteria up to date [32].

4. Regulation and Control of Enzyme Genes in Fermentative Hydrogen Production:

4.1. Hydrogenase Gene:

Hydrogen-producing microbes are a special hydrogen metabolic system compared with non hydrogen-producing microbes. Hydrogenase, the last rate-limiting enzyme of hydrogen release during the process of energy metabolism, plays a key role in hydrogen-producing metabolism [33]. Expression activity and period of hydrogenase may directly affect the energy metabolism of hydrogen-producing bacteria, and thereby influence the release rate and yield of hydrogen [24].

The over expression of hydrogenase is beneficial to the increase of hydrogen-producing rate. Miyake et al. [34] transformed Fe hydrogenase gene of *Clostridium pasteurianum* ATCC6013 into *Synechococcus elongates* PCC7942, and realized heterogeneous expression at the action of strong promoter, which was three times higher than wild type in hydrogen-producing rate. Some researchers transformed hydrogenase gene of bacteria into wild bacteria to form transgenic bacteria and made hydrogenase gene to be unexpressed. The result indicated that the hydrogen yield of transgenic bacteria was higher than that of wild type bacteria [35]. Morimoto et al. [36] reported that over expression of a hydrogenase gene in *Clostridium paraputrificum* may enhance hydrogen gas production. A [Fe]-hydrogenase gene (*hydA*) was cloned from *C. paraputrificum* M-21 in *Escherichia coli* using a conserved DNA sequence of clostridial hydrogenase genes amplified by PCR as the probe. It was ligated into a shuttle vector, pJIR751, originally constructed for *Clostridium perfringens* and *E. coli*, and expressed in *C. paraputrificum*. Hydrogen gas productivity of the recombinant increased up to 1.7-fold compared with the wild type.

Gorwa et al. [37] analyzed the molecular characterization and transcription of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824. They found that the 1745 bp *hydA* encoded a 64,415 Da protein and presented strong identity with the [Fe] hydrogenase genes of *Desulfovibrio* and *Clostridium* species. Menon et al. [38] cloned, sequenced, and analyzed mutation of the *hyb* operon encoding *E. coli* hydrogenase 2 (HYD2). Their result showed that the genes encoding the two structural subunits of *E. coli* hydrogenase 2 (HYD2) occurred in an operon (*hyb*) which contained seven open reading frames. All seven open reading frames were required for restoration of wild type levels of active HYD2 in AP3. Mishra et al. [39] isolated a novel [Fe]-hydrogenase gene from a high rate of hydrogen-producing *Enterobacter cloacae* IIT-BT 08 and carried out over expression of [Fe]-hydrogenase gene.

Xu et al. [40] isolated the *hupR* genes from the photosynthetic bacterium *Rhodobactersphaeroides*, the *hupR* gene was sequenced (EMBL Accession No. AJ243734). It encodes a 54.031 kDa protein homologous to transcriptional regulators belonging to the superfamily of two-component regulatory systems. The HupR protein was overexpressed in *E. coli* in the form of His6-tagged HupR. The cloned *hupR* gene could restore hydrogenase activity in *R. sphaeroides* *hupR* mutants and activate *hupSL* gene transcription.

4.2. Other Regulations and Controls in Fermentative Hydrogen-Producing Metabolism:

Yoshida et al. [41] performed the genetic recombination for the strain SR13 of *E. coli* to elevate the efficiency of hydrogen production by biotechnology. The formate hydrogen lyase (FHL)-over expressing strain SR13 was constructed by combining FHL repressor (*hycA*) inactivation with FHL activator (*fhlA*) over expression. Transcriptions of large-subunit formate dehydrogenase, *fdhF*, and large-subunit hydrogenase, *hycE*, in strain SR13 increased 6.5- and 7.0-fold, respectively, compared with the wild type strain. On its own, this genetic modification effectively resulted in a 2.8-fold increase in hydrogen productivity of SR13 compared with the wild type strain. Further enhancement of productivity was attained by using a novel method involving the induction of the FHL complex with high-cell-density filling of a reactor under anaerobic conditions. Continuous hydrogen production was achieved by maintaining the reactor concentration of the substrate (free formic acid) less than 25 mM. Gonzalez-Pajuelo et al. [42] studied the metabolic engineering strategy of *Clostridium acetobutylicum* for the industrial production of 1, 3-propanediol from glycerol. The 1, 3-propanediol

pathway from *C. butyricum* was introduced on a plasmid in several mutants of *C. acetobutylicum* altered in product formation, and the pSPD5 recombinant strain was further characterized from a physiological and biotechnological point of view. Chemostat cultures of this strain grown on glucose alone produced only acids (acetate, butyrate and lactate) and a high level of hydrogen.

5. Prospect of Anaerobic Fermentative Biohydrogen Production:

At present, genome sequencing of several species has been completed, and full genome sequencing of some microbes, animals, and plants has been performed, making the research of microbial molecular breeding increasingly promising. A few studies on microbial fermentative hydrogen production were to artificially regulate and control cell metabolic pathway to enhance hydrogen-producing efficiency at a microbial molecular level. In order to realize industrial production of microbial fermentative hydrogen production and release the shortage of mankind resources, the future research needs to be further developed in the following areas:

5.1. Developing New Techniques for Breeding Hydrogen-Producing Bacteria:

Besides *E. coli*, there are many microbes serving as the host bacteria of genetic engineering, researchers have established transformation and expression system of some Gram positive bacteria, such as lactic acid bacteria, bacillus, and so on. Presently, many new techniques and instruments have been successively developed. For example, gene chip, microarray, real-time quantitative polymerase chain reaction, protein two-dimensional electrophoresis, multidimensional liquid chromatography, and surface plasmon resonance are playing a promoting role in microbial molecular biology and microbial molecular breeding [43]. However, some transformation and expression systems of fermentative hydrogen-producing microbes are still imperfect, and the study on molecular breeding aiming at hydrogen-producing fermentation is still in the initial stages. Therefore, developing new breeding methods of hydrogen-producing fermentative microbe is a major task.

5.2. Exploitations of More Strains and Gene Resources:

Molecular breeding techniques of *E. coli*, *B. subtilis*, actinomycete, yeast, and filamentous fungus have been widely applied [43]. However, only a few microbial species could produce hydrogen, such as *Bacteroides*, *Zymomonas*, *Clostridium*, and *Fusobacterium*. The basis of molecular breeding of these bacteria was rather weak, thus limiting the exploitation of recombinant engineering bacteria. From this point of view, it is an important study content of biohydrogen production that further helps discover and enlarge the scope of hydrogen-producing microbial strains. In recent years, the study on extreme microorganism and extreme enzyme made a rapid development all over the world, and researchers had obtained many kinds of bacteria strains with enzyme exploitation value from ocean extreme microorganism. American researchers isolated more than 1.21×10^6 genes, exceeding the total of microbe gene in public database, with the new genes of more than 7×10^4 , of these, the function of about 5×10^3 genes was related to the exploitation of hydrogen energy [43].

5.3. Intensifying the Application of Microbial Molecular Breeding in Hydrogen Production:

Presently, microbial fermentative hydrogen production has relatively matured in fermentation technology and technique flow. However, hydrogen-producing efficiency is still low. Thus, it is important to switch study directions to recombine microbes to enhance its hydrogen-producing efficiency by modern biotechnology of metabolic engineering, genetic engineering, and protein engineering.

5.4 Metabolic Engineering:

Metabolic engineering is a promising approach to the improvement of biological hydrogen production by existing microorganisms, particularly as concerns the redirection and optimization of the flow of reducing equivalents to the H₂-producing enzymes (nitrogenase or hydrogenase). Its application, however, will require a detailed understanding of the metabolic pathways and regulatory circuits involved, many of which are extremely complex. Fig. 3 indicates how this approach may be integrated into the overall scheme of biological H₂ production, in combination with other approaches, such as genomics and biodiversity studies, as well as the emerging sciences of metagenomics [26] and systems biology

5.5 Molasses in Activated Sludge Immobilized Bio Reactor:

A feasible cell immobilization method for entrapment of activated sludge for anaerobic hydrogen production from molasses. Acclimation of the immobilized sludge via continuous operation was required in upgrading the performance of H₂ production. Organic loading rate (OLR) was shown to significantly affect the effectiveness of H₂ fermentation. The maximum hydrogen production rate of 3.65 L was obtained in the reactor at OLR of 24 kg/m³ d. The immobilized H₂-producing biocatalyst developed here may have potential for practical applications for conversion of carbohydrate-bearing wastewater into clean energy (H₂).

Conclusion:

Biological H₂ production is the most challenging area of biotechnology with respect to environmental problems. A challenging problem in establishing biohydrogen as a source of energy is the renewable and environmentally friendly generation of large quantities of H₂ gas. However, two major aspects need indispensable optimization, viz., a suitable renewable biomass/wastewater and ideal microbial consortia that can convert this biomass efficiently to H₂. Comparative studies on the available processes indicate that biohydrogen production requires greater improvement on the process mainly with respect to H₂ yield from the cheaper raw materials. The future of biological H₂ production depends not only on research advances, i.e., the improvement in efficiency through genetically engineered microorganisms and/or the development of bioreactors, but also on economic considerations (the cost of fossil fuels), social espousal and the development of H₂ energy systems.

References:

1. X.H. Xing, C. Zhang Research progress in dark microbial fermentation for biohydrogen production *Chin J Bioprocess Eng*, 3 (1) (2005), pp. 1–8 [in Chinese]
2. N.Q. Ren, M. Lin, X.P. Ma, et al. A strain of anaerobic bacteria screened for high efficient hydrogen production and its aciduric character *Acta Energetica Solaris Sinica*, 24 (1) (2003), pp. 80–84 [in Chinese]
3. Y.F. Li, N.Q. Ren, J. Ding, et al. Biohydrogen: II. The engineering and applications. *Adv Earth Sci*, 19 (Suppl.) (2004), pp. 542–546 [in Chinese]
4. J.Z. Li, N.Q. Ren The operational controlling strategy about the optimal fermentation type of acidogenic phase *China Environ Sci*, 18 (5) (1998), pp. 398–402 [in Chinese] View Record in Scopus | Citing articles (16)
5. H. Zhang, M.A. Bruns, B.E. Logan Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor *Water Res*, 40 (4) (2006), pp. 728–734 Article | PDF (317 K) | View Record in Scopus | Citing articles (133)

6. K.S. Lee, J.F. Wu, Y.S. Lo, et al. Anaerobic hydrogen production with an efficient carrier-induced granular sludge bed bioreactor *Biotechnol Bioeng*, 87 (5) (2004), pp. 648–657
7. View Record in Scopus | Full Text via CrossRef | Citing articles (150) A. Perra, E.S. Davies, J.R. Hyde, et al. Electrocatalytic production of hydrogen by a synthetic model of [NiFe] hydrogenases *Chem Commun (Camb)* (10) (2006), pp. 1103–1105 View Record in Scopus | Full Text via CrossRef | Citing articles (35)
8. W. Zhang, J.E. Zuo, L.T. Cui Continuous biohydrogen production by mesophilic and thermophilic cultures *Environ Sci*, 27 (1) (2006), pp. 63–68 View Record in Scopus | Full Text via CrossRef | Citing articles (1)
9. N.Q. Ren, A.J. Wang, F. Ma Acid-producing fermentative microbe physiological ecology Science Press, Beijing (2005) [in Chinese]
10. J. Zuo, Y. Zuo, W. Zhang, et al. Anaerobic bio-hydrogen production using pre-heated river sediments as seed sludge *Water Sci Technol*, 52 (10) (2005), pp. 31–39 View Record in Scopus | Citing articles (13)
11. S.H. Kim, S.K. Han, H.S. Shin Performance comparison of a continuous-flow stirred-tank reactor and an anaerobic sequencing batch reactor for fermentative hydrogen production depending on substrate concentration *Water Sci Technol*, 52 (10–11) (2005), pp. 23–29 View Record in Scopus | Full Text via Cross Ref | Citing articles (15)
12. M. Lin, N.Q. Ren, A.J. Wang, et al. Cooperation of mixed culturing bacteria in the hydrogen production by fermentation *Environ Sci*, 24 (2) (2003), pp. 54–59 [in Chinese] View Record in Scopus | Citing articles (5)
13. J. Lay Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose *Biotechnol Bioeng*, 74 (4) (2001), pp. 280–287 View Record in Scopus | Citing articles (198)
14. M. Lin, N.Q. Ren, X.P. Ma, et al. Selection and improvement of culture for hydrogen producing bacteria *J Harbin Inst Technol*, 35 (4) (2003), pp. 398–402 [in Chinese] View Record in Scopus | Citing articles (19)
15. Y. Wang, N.Q. Ren, Y.J. Sun Analysis of the ferment process and pro-hydrogen ability of pro-hydrogen and ferment bacterium influenced by *FeActa Energiae Solaris Sinica*, 24 (2) (2003), pp. 222–226 [in Chinese]
16. N.Q. Ren, J.X. Song, D. An, et al. Effects of terminal products on hydrogen production by ethanol hydrogen-producing microflora *Environ Sci*, 27 (8) (2006), pp. 1608–1612 [in Chinese]
17. S. van Ginkel, B.E. Logan Inhibition of biohydrogen production by undissociated acetic and butyric acids *Environ Sci Technol*, 39 (23) (2005), pp. 9351–9356 View Record in Scopus | Full Text via Cross Ref | Citing articles (118)
18. T. Kurokawa, S. Tanisho Effects of formate on fermentative hydrogen production by *Enterobacter aerogenes* *Mar Biotechnol (NY)*, 7 (2) (2005), pp. 112–118 View Record in Scopus | Full Text via CrossRef | Citing articles (29)
19. M.B. Salerno, W. Park, Y. Zuo, et al. Inhibition of biohydrogen production by ammonia *Water Res*, 40 (6) (2006), pp. 1167–1172 Article | PDF (277 K) | View Record in Scopus | Citing articles (82)
20. Y.F. Li, N.Q. Ren, J.Z. Li, et al. Isolation and identification of several hydrogen-producing fermentative bacteria in biohydrogen reactor West exploitation and sustainable development – environmental protection – the ninth Mainland and Taiwan Environmental Protection Forum, Xi’an Jiaotong University Press (2004), pp. 830–834 View Record in Scopus

21. J.Z. Li, N.Q. Ren, M. Lin, et al. Hydrogen bio-production by anaerobic fermentation of organic wastewater in pilot-scale *Acta Energetica Sinica*, 23 (2) (2002), pp. 252–256 [in Chinese] Record in Scopus | Citing articles (12)
22. Y.G. Cui, Y.F. Li, N.Q. Ren, et al. The techniques of genetic mutation for bacterium on bio-hydrogen production by beet sugar molasses *China Beet Sugar*, 3 (2004), pp. 1–4 [in Chinese]
23. P. Iyer, M.A. Bruns, H. Zhang, et al. H₂-producing bacterial communities from a heat-treated soil inoculums *Appl Microbiol Biotechnol*, 66 (2) (2004), pp. 166–173 View Record in Scopus | Full Text via CrossRef | Citing articles (76)
24. Q.B. Li, D.F. Xing, N.Q. Ren, et al. Hydrogen production and enzyme activity of acidophilic strain X-29 at different c/n ratio *Environ Sci*, 27 (4) (2006), pp. 810–814 [in Chinese] View Record in Scopus | Citing articles (4)
25. Y.F. Li, N.Q. Ren, C.P. Yang, et al. Preliminary appraisal and H₂-producing characters of a strain highly efficient H₂-producing and acid-producing Bacterium *China Environ Sci*, 25 (2) (2005), pp. 210–213 [in Chinese]
26. Y.F. Li, N.Q. Ren, L.J. Hu, et al. Characterization and sequence analysis of 16S rDNA of a new species of high efficient biohydrogen production anaerobe *J Harbin Inst Technol*, 37 (3) (2005), pp. 287–289 [in Chinese]
27. Q.Q. Shi, S.G. Wu *Industrial microbe breeding* Science press, Beijing (2003) [in Chinese]
28. Y.F. Li, N.Q. Ren, L.J. Hu, et al. Biohydrogen processes and H₂-producing mechanism *Energy Environ Protect*, 19 (2) (2005), pp. 5–7 [in Chinese]
29. N.Q. Ren, L.Y. Xu, Y. Zhang, et al. Dependence on iron and hydrogen producing pathway for novel strain *E. thanoligenens* sp. B49 *Acta Scientiae Circumstantia*, 26 (10) (2006), pp. 1643–1650 [in Chinese]
30. T. Noike, I.B. Ko, S. Yokoyama, et al. Continuous hydrogen production from organic waste *Water Sci Technol*, 52 (1–2) (2005), pp. 145–151
31. D.F. Xing, N.Q. Ren, Q.B. Li, et al. *Ethanoligenens harbinense* gen nov., sp. Nov., isolated from molasses wastewater *Int J Syst Evol Microbiol*, 56 (4) (2006), pp. 755–760
32. D.F. Xing, Study on community structure and function of hydrogen-producing and ethanol-producing bacteria [in Chinese]. Ph.D. Dissertation, Harbin Institute of Technology; 2006.
33. Z.Y. Zhu, J.A. Yu, Z.X. Lin, et al. Study and application of photosynthetic bacteria *Shanghai Jiaotong University Press*, Shanghai (1991) [in Chinese]
34. J. Miyake, T. Matsuura, A.S. Pietro *Biohydrogen II: an approach to environmentally acceptable technology* Elsevier Science LTD (2001), pp. 205–219
35. K. Ohmiya, K. Sakka, T. Kimura, et al. Application of microbial genes to recalcitrant biomass utilization and environmental conservation *J Biosci Bioeng*, 95 (6) (2003), pp. 549–561 Article | PDF (1985 K) | View Record in Scopus | Full Text via Cross Ref | Citing articles (29)
36. K. Morimoto, T. Kimura, K. Sakka, et al. Over expression of a hydrogenase gene in *Clostridium paraputrificum* to enhance hydrogen gas production *FEMS Microbiol Lett*, 246 (2) (2005), pp. 229–234
37. M.F. Gorwa, C. Croux, P. Soucaille Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824 *J Bacteriol*, 178 (9) (1996), pp. 2668–2675

38. N.K. Menon, C.Y. Chatelus, M. Dervartanian, et al. Cloning, sequencing, and mutational analysis of the h₂ operon encoding Escherichia coli hydrogenase J Bacteriol, 176 (14) (1994), pp. 4416–4423
39. J. Mishra, S. Khurana, N. Kumar, et al. Molecular cloning, characterization, and over expression of a novel [Fe]-hydrogenase isolated from a high rate of hydrogen producing Enterobacter cloacae IIT-BT 08 Biochem Biophys Res Commun, 324 (2) (2004), pp. 679–685 Article | PDF (1395 K) | View Record in Scopus | Citing articles (33)
40. D.Q. Xu, Y.Q. Wu Isolation and analysis of hup gene required for the expression of hydrogenase in Rhodospirillum rubrum Acta Biochim Biophys Sinica, 33 (6) (2001), pp. 607–614 [in Chinese]
41. Yoshida, T. Nishimura, H. Kawaguchi, et al. Enhanced hydrogen production from formic acid by formate hydrogen lyase-overexpressing Escherichia coli strains Appl Environ Microbiol, 71 (11) (2005), pp. 6762–6768
42. M. Gonzalez-Pajuelo, I. Meynial-Salles, F. Mendes, et al. Metabolic engineering of Clostridium acetobutylicum for the industrial production of 1,3-propanediol from glycerol Metab Eng, 7 (5–6) (2005), pp. 329–336
43. Fermentative Hydrogen Production from Molasses in an Activated Sludge Immobilized Bioreactor IJEE Vol. 2 No. 1, 2012 PP. 28-31 www.ij-ee.org