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Review

Recent advances in fermentative biohydrogen production

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Abstract

Hydrogen energy, as a kind of clean energy with great potential, has been a hotspot for study worldwide. Based on the recent research on biohydrogen production, this paper gives a brief review 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. Moreover, 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.

Keywords: Fermentation; Biohydrogen production; Regulation and control; Novel strains

1. Introduction

Fossil energy source cannot be regenerated and will be exhausted with increasingly more fossil fuel consumptions. 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.

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2. Fermentative hydrogen production process and the engineering control statagy

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, metacetonic acid-type fermentation, and ethanol-type fermentation. In parallel, there are three acid-producing fermentation floras: butyric acid-type fermentation flora, metacetonic 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 metacetonic 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 (H_2/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/m^3 d [9]. Zuo et al. [10] used pre-heated river sediments as seed sludge to conduct anaerobic biohydrogen 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/m^3 d, the highest hydrogen production was 6.3–6.7 L H_2/L reactor d, the specific hydrogen production was 1.3–1.4 mol H_2/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 (So/Xo) [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 Mg^{2+} can promote the growth of ethanol type hydrogen-producing fermentative bacteria and enhance its hydrogen-producing ability.

2.2.4. Anaerobic fermentation terminal products

Anaerobic fermentation terminal 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 [4,21], 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* (subcluster Ic) at 30 °C to populations related to *Clostridium acetobutylicum* (subcluster Ib).

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 [2,28]. 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-3^T 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-3^T and X-29 represent a distinct genus within the *Clostridium leptum* rRNA subgroup. Its taxonomic name is *Fthanoligeneras harhiraense* Gen.Nov.Sp.Nov. Only one species, *Fthannligenens harhinense*, has been described so far, and this species has been designated the type species. The type strain is YUAN-3^T, which formed large auto-aggregative granule on shake culture. Specific hydrogen conversion rate and hydrogen production rate of strain YUAN-3^T were 2.81 mol H_2/mol-glucose and 27.6 mmol H_2/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 overexpression 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]. Morinoto et al. [36] reported that overexpression 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 overexpression of [Fe]-hydrogenase gene.

Xu et al. [40] isolated the *hupR* genes from the photosynthetic bacterium *Rhodobacter sphaeroides*, 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)-overexpressing strain SR13 was constructed by combining FHL repressor (*hycA*) inactivation with FHL activator (*fhlA*) overexpression. 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 recombined 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.

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