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Effect of temperature and iron concentration on the growth and hydrogen production of mixed bacteria

Yongfang Zhang, Jianquan Shen*

The Chinese Academy of Sciences, Institute of Chemistry, Zhongguancun, Beijing 100080, PR China

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Abstract

Anaerobic mixed culture acclimated with sucrose was used as inoculum in batch experiments to investigate the effects of various parameters on biological hydrogen production from sucrose. In particular, the effect of the culture temperature has been investigated in detail. The optimum of the iron concentration in the external environment on hydrogen production was also studied at different temperatures. Experimental results show that the hydrogen production ability of the anaerobic bacteria was deeply affected by both culture temperature and iron concentration. Increasing the culture temperature favored the production of hydrogen when it was in the range of 25–40 °C, and high sucrose conversion efficiencies (ca. 98%) were consistently obtained with the mixed bacteria at the same time. While the temperature went on increasing to 45 °C, the hydrogen production was almost inhibited. The optimum concentrations of iron for hydrogen production decreased obviously along with increasing the reactor's temperature. For 25, 35, and 40 °C, the maximum production yield of hydrogen were 356.0, 371.7, and 351.1 ml obtained at the iron concentration of 800, 200, and 25 mg $FeSO_4$ l^{-1}, respectively.

Keywords: Sucrose fermentation; Hydrogen production; Culture temperature; Iron concentration

1. Introduction

Methane and hydrogen are important gaseous fuels produced by the anaerobic fermentation of organic waste [1]. However, methane and its combustion product carbon dioxide are both greenhouse gases; hence, more efforts are being focused on the development of clean energy sources. Hydrogen is a promising energy alternative. It is clean, renewable, and generates no toxic byproduct, water [2]. It has a high-energy content per unit weight (122 kJ g^{-1}) and thus is an attractive energy source for replacing conventional fossil fuels, both from the economic and environmental standpoints [3]. At present, despite the "green" nature of hydrogen as a fuel, it is still primarily produced from nonrenewable sources such as natural gas and petroleum hydrocarbons via steam reforming. Therefore, if hydrogen is to replace fossil fuels in the future, it has to be produced renewably and in large scale, with environmentally benign processes [4]. Microbial hydrogen production using fermentative bacteria, photosynthetic bacteria, cyanobacteria, or algae is an environmentally friendly and energy saving process. It is more attractive if organic wastewater or other wastes are used as the raw material. This economical bioenergy-producing process can produce an energy product and simultaneously reduce the pollution strength of the wastes [5].

Although the conversion of biomass resources to hydrogen gas by fermentation has been extensively studied, most of these studies have been carried out with laboratory-scale reactors using pure cultures of isolated strains. Detailed information regarding temperature control of bioreactors for efficient hydrogen production is still lacking, especially for the systems associated with mixed cultures. However,

* Corresponding author. Fax: +86 10 625 593 73.
E-mail address: jqshen@iccas.ac.cn (J.Q. Shen).

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before moving on to even larger volumes with outdoor operation it is necessary to determine and evaluate the distinct effects of as many parameters as possible. In general, factors determining optimal hydrogen production, such as pH, temperature, concentration of electron donor, age of culture, cell density, and nutritional history of the cells, may differ from optimal factors for growth [6]. In these factors environmental temperature is one of the most important which affect both the cultivation and the hydrogen-producing activity of microorganisms. Consequently, it would influence the production yield of hydrogen from residual organic substrates in connection with wastewater treatment. To better understand the factors that can affect hydrogen production, two aspects relative to hydrogen production were examined by mixed cultures. First, we measured biohydrogen production in four conditions of batch tests that differ by the temperatures of reactors. Second, to investigate the influence of iron limitation on sucrose fermentation, cells were grown at 25–3200 mg l–1 ferrous ions present in the medium, and product formation was compared with the blank test at the four temperatures, respectively.

We isolated microorganisms from the cracked cereal that demonstrated stable and high-yield production of hydrogen from glucose, sucrose, starch, and other carbohydrates in a chemostat culture. The main purpose of this paper is to show to what extent the temperature may affect the values of fermentation parameters calculated from results obtained in batch experiments using the above-mentioned mixed bacteria. We selected mixed bacteria, not pure ones, mainly because of their ability to consume organic substrates derivable from wastes and then, for their potential to be used in association with wastewater treatment. The data obtained from this study are expected to provide basic and engineering data for the bioreactor design of practical bioprocesses aimed to produce stable and prolonged hydrogen from wastewater.

2. Materials and methods

Unless noted otherwise, reagents and solvents were analytical pure and purchased from Beijing Yili Fine Chemical Ltd. Co., China.

2.1. Inoculum

The anaerobic bacteria used in this study were enriched from cracked cereals brought to a 2 h bake and a 30 min boil to inhibit the bioactivity of hydrogen consumers. The cereals were seeded into a 1–l completely stirred tank reactor (CSTR), grown on a sucrose mineral salts medium at 35 °C and a 24 h hydraulic retention time (HRT), and stirred by gas circulation. The organisms responsible for hydrogen production were dominated by *Clostridium pasteurianum*. The 500 ml culture was used to inoculate 1–l of grown medium and after 1 month's growth it could be used. One liter of the medium contained sucrose, 11,100 mg; NH_4HCO_3, 3770 mg; K_2HPO_4, 125 mg; Na_2CO_3, 2000 mg; $CuSO_4{\cdot}5H_2O$, 5 mg; $MgCl_2{\cdot}6H_2O$, 100 mg; $MnSO_4{\cdot}4H_2O$, 15 mg; $FeSO_4{\cdot}7H_2O$, 25 mg; $CoCl_2{\cdot}6H_2O$, 0.125 mg.

2.2. Experimental procedures

Batch experiments were conducted in 120-ml vials with a working volume of 80 ml. After addition of 10 ml inoculum, 50 ml nutrient solution (prepared by adding sucrose, 40,000 mg; NH_4HCO_3, 16,000 mg; $NaHCO_3$, 4000 mg; $K_2HPO_4{\cdot}3H_2O$, 250 mg; $CuSO_4{\cdot}5H_2O$, 10 mg; $MgCl_2{\cdot}6H_2O$, 200 mg; $MnSO_4{\cdot}4H_2O$, 30 mg; $CoCl_2{\cdot}6H_2O$, 0.25 mg into 1 l of distilled water), and 20 ml of the $FeSO_4$ solution (ranging from 0 to 12,800 mg $FeSO_4$ l^{-1}). The total volume of liquid in the bottles was 80 ml, which resulted in a solution with a concentration of 25 g sucrose l^{-1}. The concentration of $FeSO_4$ ranged from 0, 25, 100, 200, 400, 800, 1600, 2400 to 3200 mg l^{-1} in the batch experiment, and it excluded the residual iron concentration in the inoculum. The air was removed from the headspace by Argon gas sparging for 5 min before the bottles were capped with rubber septum stoppers and placed in a reciprocal shaker (Reciprocation: 5 cm × 120 strokes per min). The batch experiments were conducted in the dark at 35 °C. The solutions were adjusted to an initial pH = 8 with 1 M HCl or 1 M NaOH. The biogas production was measured by a plunger displacement method using appropriately sized glass syringes, arranging from 10 to 100 ml [7]. Each experimental condition was carried out in triplicate.

2.3. Chemical analysis

The proportion of hydrogen was determined with a gas chromatograph (SHIMADZU 14B, Japan) equipped with a thermal conductivity detector (TCD). A 2-m stainless iron column packed with Porapak Q (50/80 mesh). Argon was used as the carrier gas at a flow rate of 30 ml min^{-1}. The operating temperatures of the injection port, the oven, and the detector were 80, 50 and 100 °C, respectively. Detection of the volatile fatty acids (VFAs, C_2–C_4) was also analyzed by a gas chromatograph (SHIMADZU 14B, Japan) using a flame ionization detector (FID). A 2-m glass column packed with Unisole F-200 (30/60 mesh). The temperatures of the injection port, the oven, and the detector were 200, 150, and 220 °C, respectively. The carrier gas was also Argon at a flow rate of 30 ml min^{-1}. The concentrations of sucrose after the reaction were determined by the phenol–sulfuric acid method using sucrose as a standard [8]. The concentrations of volatile suspended solids (VSS) were determined according to the procedures described in the standard methods [9]. The pH was measured using a pH meter (PHS-3B Shanghai, China). All gas production data reported were standardized to standard temperature (0 °C) and pressure (760 mmHg).

3. Results and discussion

3.1. Effect of temperature

A set of experiments was designed to investigate the effects of culture temperature on hydrogen formation and cell growth in batch cultures of mixed bacteria with different iron concentrations. Fig. 1 shows the concentration courses of hydrogen production yield, hydrogen percentage, cell concentration VSS, and pH value obtained with various temperatures.

3.1.1. Effect of temperature on hydrogen production and hydrogen percent

Fig. 1a illustrates the influence of different temperatures on hydrogen production at different iron concentration. For the tests at 25 °C, the hydrogen production yield obviously increased with the increase of iron concentration until it reached 800 mg $FeSO_4\,l^{-1}$, and then decreased with the following increase of iron. For 35 °C, the normal temperature, the hydrogen yield increased with increasing iron concentration to 200 mg $FeSO_4\,l^{-1}$ and decreased subsequently. For 40 °C, the hydrogen yield reached the peak at 25 mg $FeSO_4\,l^{-1}$, very low iron concentration. Whereas hydrogen production was almost inhibited for the tests at 45 °C. The bacteria nearly did not evolve molecular hydrogen under this condition. For 25, 35, and 40 °C, the maximum production yield of hydrogen were 356.0, 371.7, and 351.1 ml obtained at the iron concentration of 800, 200, and 25 mg $FeSO_4\,l^{-1}$, respectively.

Product analysis shows that the anaerobic fermentation produced a biogas only containing hydrogen and carbon dioxide, without detectable methane. The percentage of hydrogen in the biogas fluctuated with the variations of both temperature and iron concentration, which ranged from 4.7 to 42.9% at the tested temperatures shown in Fig. 1b. According to this figure, the maximum hydrogen percent were 41.7, 42.0, and 42.9% obtained at 800, 200, and 25 mg $FeSO_4\,l^{-1}$ for 25, 35, 40 °C, respectively, which has the same trend with the maximum hydrogen production. However, for the system of 45 °C, the hydrogen percent decreased obviously with the increase of iron concentration.

3.1.2. Effect of temperature on cell growth and pH

Fig. 1c shows the variation of VSS that fluctuates obviously with the increase of ambient temperature. It also shows that the dry cell weight had exceptional differences with the variation in iron concentration. For 25 °C, VSS of all the tests had great difference from each other with the variation of the iron concentration, which increased to about 2100 mg l^{-1} at 200 mg $FeSO_4\,l^{-1}$ and then remained high due to the increase of iron concentration. For the other three, the cell concentrations were kept steady for each and almost did not change for the variation of iron concentrations.

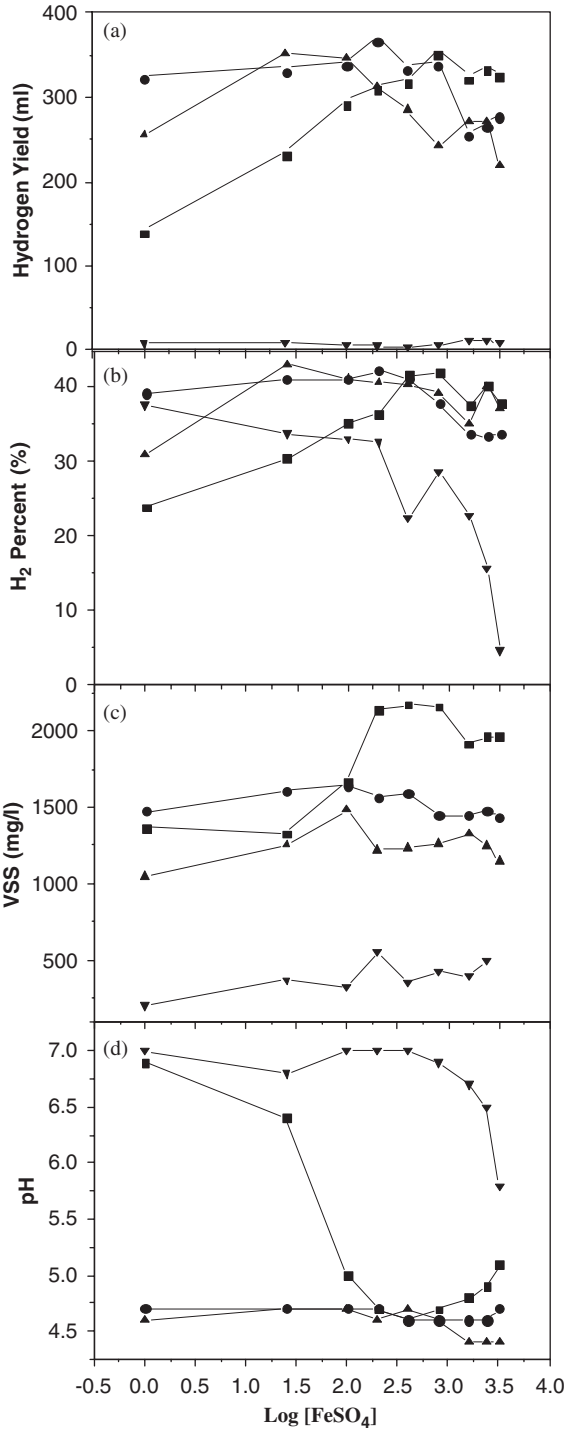

Fig. 1. Comparison of (a) hydrogen yield (ml), (b) hydrogen percent (%), (c) the dry cell weight (VSS, mg/l) and (d) the ultimate pH value measured at 25 °C (■), 35 °C (●), 40 °C (▲), and 45 °C (▼), respectively. Values shown are averages of bottles run in triplicate.

The ultimate pH values of the medium are shown in Fig. 1d. For 25 °C, the pH dropped sharply from 6.9 to 4.6 as the iron concentration varied from 0 to 3200 mg $FeSO_4\,l^{-1}$. For 35 and 40 °C, the pH values are almost invariable, dropped from an initial value of 8.0 to about 4.7. As mentioned above, the hydrogen production of the bacteria was almost inhibited at 45 °C, so the medium pH values under these conditions were about 7.0, relatively high. It is indicated that with the increase of the concentration of iron, the decrease of pH values at 25 °C is the most obvious one among those at 35, 40, and 45 °C which is possibly because the amount of the metabolic product is different at different temperatures. The hypothesis could also be verified by the results of the VFAs production as shown in Fig. 2a.

3.1.3. Effect of temperature on production of VFAs and solvent

The formation of hydrogen is accompanied with VFAs or solvent production during an anaerobic digestion process. Therefore, the VFA concentration distributions and their fractions are useful indicators for monitoring hydrogen production. Fig. 2 shows the profile of soluble metabolites production during the course of batch hydrogen fermentation. It can be seen from the distribution of solvent products in Fig. 2 that for most tests the soluble metabolites principally consisted of ethanol, acetic acid, and butyric acid, while a relatively smaller amount of propionic acid was also detected. In this study, low hydrogen production occurred at the blank test of 25 °C, and its liquid product displayed immense difference from that of other tests (Fig. 2a). Low ambient temperature and iron limitation had a pronounced effect on sucrose fermentation; significant amounts of propionate were produced, which became the major product (7380 mg l^{-1}) at the blank test, mostly at the expense of acetate and butyrate. A marked variation in liquid production fractions indicates a shift in the metabolic pathway in an anaerobic digestion process. Because the metabolic pathway relates to the bioactivity of the dominant microorganisms, the experimental results show the temperature-dependent characteristic of the biomass activity. The main difference between the tests of four temperatures was an obvious increase in ethanol concentration from about 550 to 3600 mg l^{-1} or so. It indicated that a major shift in metabolic pathways attributed to the variation of temperatures, i.e. from acids to solvent production. It has been suggested that the switch to solvent production is an adaptive response of the cell to the low-medium pH resulting from acid production [10,11]: solvent production usually occurs only after the external pH falls below 5.0. During the exponential phase of growth, the mixed bacteria produced over 100 mM total acids (acetic, propionic, and butyric). As a result, the pH of the medium dropped from an initial value of 8.0 to about 4.7. Iron limitation somehow inhibited the formation of ethanol. It is possible that the alcohol dehydrogenases involved are iron dependent [12]. What controls the switch to solvent

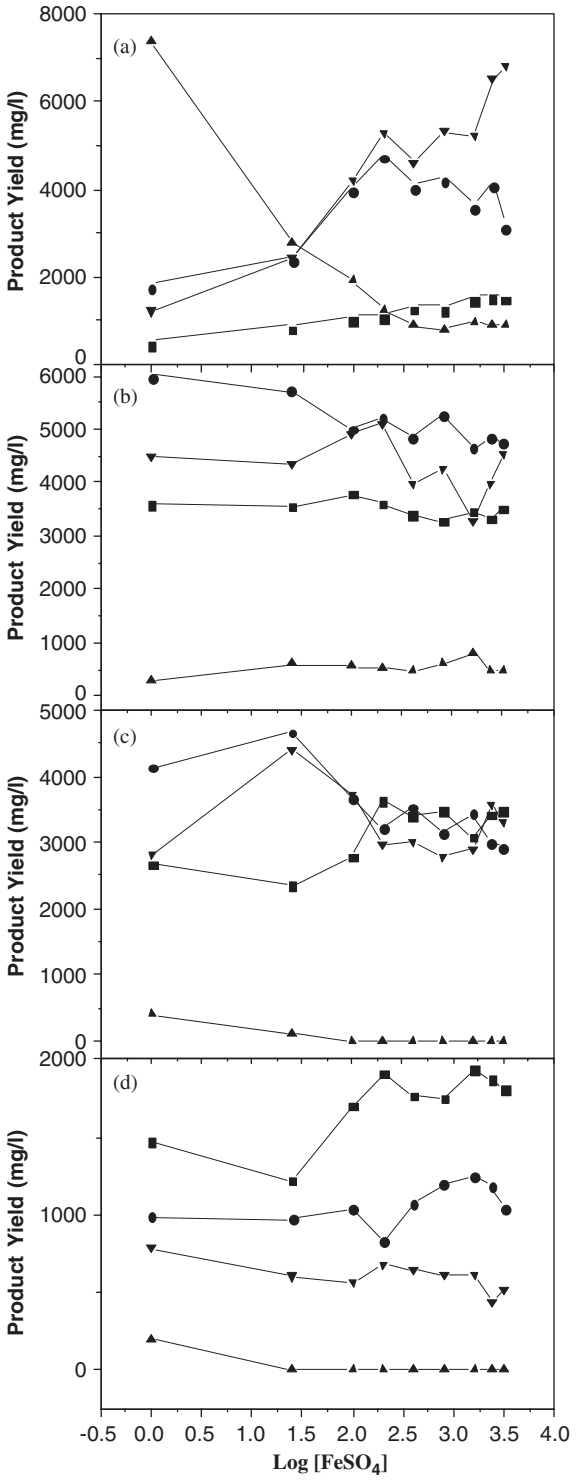

Fig. 2. The production yield of ethanol (■), acetic acid (●), propionic acid (▲), and butyric acid (▼) measured at (a) 25 °C, (b) 35 °C, (c) 40 °C, and (d) 45 °C, respectively. Values shown are averages of bottles run in triplicate.

production has not been determined in detail because the regulation of hydrogen/solvent production is complex and dependent on more than one factor such as pH, iron limitation, carbon source, phosphate as well as seed microorganisms [12,13].

Not all of the tests were observed to produce hydrogen immediately after inoculum. For 25, 40, and 45 °C, the occurrence of the lag phase, a phase without any gas production was observed (data not shown). A long lag phase of ca. 36 h was found at 25 °C, and longer lag phases of ca. 72 h and ca. 96 h were observed at 40 and 45 °C, respectively. A possible factor contributing to the lag phase appeared at 25, 40, and 45 °C, compared with the normal 35 °C, could be that the mixed bacteria need more time to adapt to the new environment with unusual temperatures.

3.2. Concerted effect of iron ion together with culture temperature

As shown in Figs. 1 and 2, for the systems of 25 °C, there are remarkable differences between the blank and other tests. For the blank test there was a sharp decrease of both hydrogen production and the cell concentration. This probably attributed to the adaptation of cells metabolism corresponding to the initial presence of small quantities of residual iron from the inoculum and the low temperature. However, the optimum concentrations of iron for hydrogen production decreased obviously along with increasing the reactor's temperature. At 25 °C, hydrogen percent peaked at the iron concentration of 800 mg $FeSO_4\,l^{-1}$, while at 35 and 40 °C, hydrogen percent peaked at 200 and 25 mg $FeSO_4\,l^{-1}$, respectively. These findings are understandable because of two reasons. On the one hand, hydrogen evolves as the final product of reductant disposal from hydrogenase or nitrogenase activity. The primary electron donor for both enzymes is ferredoxin, which receives electrons in its turn from the reduced products of glycolysis, i.e. NADH or NADPH [14,15]. Hydrogenase present in anaerobic bacteria oxidizes reduced ferredoxin to produce molecular hydrogen [16]. We know that 35 °C or so was the optimal temperature for enzymes functioning as biochemical catalysts. Therefore, when the ambient temperature was relatively low the bacteria need more ferrous ion to activate the hydrogenase or nitrogenase so that it could oxidize reduced ferredoxin to produce more molecular hydrogen. Though the use of external iron does not enhance hydrogen production significantly, iron is obligatory for the long-term preservation of the bacteria. Without iron, the bacteria start to weaken and colonies began to lose their characteristics. On the other hand, the bacteria that we used contained hydrogen-producing bacteria as well as bacteria which do not have hydrogen production ability since they were mixed anaerobic microorganisms. Changes in the iron concentration and the ambient temperature may have affected the relationship between these bacteria and caused the variation of the metabolism.

From the results, we knew that the production of butyric acid and acetic acid was in accordance with the trend of H_2 production, indicating that acidogenic bacteria that produced butyric acid or acetic acid contributed to the majority of H_2 production. So the proliferation of acetate and butyrate producers in the microflora in order to recover hydrogen gas with high efficiency is expected. Further work is needed for the characterization of hydrogen fermentation of these dominant bacteria. The inhibition of hydrogen consumers is essential for net hydrogen production and for further scale-up and industrial application. The increase in temperature is aimed at a better performance of the hydrogenases of the thermophilic organisms, because the affinity for hydrogen decreases [17] and the thermodynamic equilibrium of hydrogen formation from e.g. acetate is favored at higher temperatures [18].

4. Conclusion

This study demonstrated that temperature affects biohydrogen production by shifting the microorganism metabolic pathway. From the above it may be clear that in the present study small increases of the culture temperature have resulted in the increase of hydrogen production, while a large increase caused inhibition in the production of hydrogen. Obtained results indicate that the sensitivity of mixed bacteria to temperature was significantly high- and the optimal-temperature for hydrogen production was around 35 °C. However, the addition of an appropriate amount of iron in batch tests enables better production of hydrogen at different temperatures. A further experiment confirmed that the results measured in this study possessed a high reliability and the mixed bacteria had a significant potential of biological hydrogen production. It appears possible that at an industrial scale, energy could be recovered as hydrogen from an acidogenic reactor as part of a typical biological treatment for carbohydrate-containing wastewaters or from an acidogenic stage used to produce volatile fatty acids for chemical or liquid biofuel production.

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