

Effects of substrate components on hydrogen fermentation of multiple substrates

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Abstract As is well known, carbohydrate is the most appropriate organic material for hydrogen fermentation, and its hydrogen yield is significantly larger than that of protein. The fermentation of protein began with hydrogen production followed by hydrogen consumption, which helps overall hydrogen recovery. Both carbohydrate and protein are basic components of organic material, and yet carbohydrate is known to be a better substrate than protein in terms of hydrogen yield during hydrogen fermentation. This study used multiple substrates containing different ratios of glucose and peptone as multiple substrates to investigate the roles played by carbohydrate and protein in hydrogen fermentation. The experimental results demonstrated that suitable ratios of glucose and peptone improved the growth of hydrogen producing bacteria. Additionally, a maximum hydrogen yield of 6.4 mmole-H₂/g-COD was obtained from the multiple substrate containing 40% peptone and 60% glucose. Most of the produced hydrogen came from fermentation of glucose, not peptone. During hydrogen fermentation, the pH dropped by 1.0 and 1.9 units in 80% and 20% of peptone content in the substrate. Ammonia produced due to peptone degradation neutralized the acids produced from hydrogen fermentation.

Keywords Biohydrogen; hydrogen fermentation; multiple substrate; substrate composition

Introduction

Hydrogen is a valuable, renewable, versatile and clean energy. Anaerobic hydrogen fermentation is one way of obtaining hydrogen. The technique of using hydrogen-fermentative bacteria to degrade organic compounds and to recover hydrogen energy from organic compounds could be applied to obtain hydrogen from organic waste or wastewater.

Most media used in previous studies on hydrogen fermentation contain protein as the nitrogen source to enhance the growth and activity of hydrogen-producing bacteria, despite carbohydrate being universally known as the most suitable organic substrate for hydrogen production (Taguchi *et al.*, 1996; Yokoi *et al.*, 1995; Ueno *et al.*, 2001). However, few studies paid attention to the importance and effects of protein on hydrogen fermentation. Most studies only focused on various influences on hydrogen production, such as substrate concentration (Ginkel *et al.*, 2001; Lay, 2001), pH (Fang and Liu, 2002; Ginkel *et al.*, 2001), partial pressure of H₂ in solution (Mizuno *et al.*, 2000), the concentration of iron (Lee *et al.*, 2001) and bacteria species.

Few investigations have noticed the importance of the substrate components. Yokoi *et al.* (1995) noticed that polypepton was highly suitable for hydrogen fermentation in various nitrogen sources. However, Yokoi also observed that polypepton would react with glucose in an autoclave to produce a chemical to inhibit hydrogen production. Ueno *et al.* (2001) demonstrated the community difference between two hydrogen fermenters, namely that one used ammonia as the sole nitrogen source while the other used ammonia plus peptone as dual nitrogen sources. Yokoi and Ueno noticed that hydrogen-fermenting bacteria need a suitable nitrogen source to improve cell growth and hydrogen production. However, being a good nitrogen source for cell growth is never the only use of protein in

hydrogen fermentation. This study conducted a systematic discussion of the effects of protein on hydrogen fermentation of multiple substrates.

Materials and methods

Organisms and culture conditions

The seeding microorganisms come from the UASB of the food industry and are pretreated with boiling for over 30 minutes to kill hydrogen consuming bacteria. However, spore formation enabled the survival of clostridia, hydrogen producing bacteria. The components of the food industry wastewater were mainly carbohydrate, protein and some lipid. Therefore the original seeding sludge must contain many carbohydrate utilizing bacteria and protein utilizing bacteria. By investigating the bacterial morphology, analyzing the 16SrDNA and checking the sequence in the gene bank, the dominative micro flora in the mixed culture used in this work was identified as clostridia (data not shown). The pretreated sludge acclimated by long-term transfer culture was then inoculated into serum bottles containing trace metals and nutrients (Table 1), 20 mM of phosphate as buffer and 5,000 mg-COD/L of multiple substrates. The multiple substrates had various glucose/peptone ratios (COD/COD), namely 0/5, 1/4, 2/3, 3/2, 4/1 and 5/0. The serum bottles were incubated in a 35°C orbital shaker and shaken at 100 rpm to achieve hydrogen fermentation. To observe the fermentation, hydrogen and carbon dioxide gas, volatile fatty acids, ammonia, MLVSS and pH were investigated.

Analysis

The pH, ORP, NH_4^+ , volatile suspended solids (VSS), components of gas production, fatty acids, alcohols, NH_4^+ and organic nitrogen were measured using Standard Methods (APHA, 1995). Moreover, biogas was analyzed using a gas chromatograph (China GC 8900, Taipei, Taiwan) equipped with a thermal conductivity detector, while a 2 m stainless column was packed with Hayesep Q (60/80 mesh) and installed inside a 60°C oven. Pure nitrogen gas was used as the carrying gas at a constant flow rate of 10 mL/min. The alcohol concentrations were determined by another GC (Shimadzu GC-14A, Kyoto, Japan) equipped with a flame ionization detector. The fatty acids were measured by ion chromatography (Dionex DX-120, California, USA) with an IonPac ICE-ASI column and conductivity detector.

Results and discussion

Hydrogen production and cell growth in the fermentation of multiple substrates

Different components of multiple substrates naturally led to hydrogen production and cell growth. The optimal hydrogen production and cell growth, which were 6.4 mmole-

Table 1 Nutrients used in hydrogen fermentation

| Trace metal | | Nutrients | |
|---|----------------------|-----------------------------|----------------------|
| Components | Concentration (mg/L) | Components | Concentration (mg/L) |
| $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ | 33.4 | Biotin | 0.002 |
| $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ | 240 | Folic acid | 0.0020.01 |
| KCl | 173.4 | Pyridoxine HCl | 0.0050.0050.00 |
| $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ | 2.66 | Riboflavin | 50.0050.0001 |
| $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ | 4 | Thiamin | 0.005 |
| H_3BO_3 | 0.76 | Pantothenic acid | 0.005 |
| $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ | 0.36 | Nicotinic acid | |
| $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ | 0.34 | Vitamin B12 | |
| ZnCl_2 | 0.28 | <i>p</i> -aminobenzoic acid | |
| $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ | 3.7 | Thioctic acid | |

H_2 /g-COD and 90 mg-MLVSS/g-COD respectively, were both observed in the fermentation of the multiple substrate component, 3,000 mg-COD/L glucose plus 2,000 mg-COD/L peptone. Too high a protein fraction or too high a carbohydrate fraction in substrate are disadvantages to hydrogen production and cell growth in hydrogen fermentation (Figure 1).

Cell growth needs enough materials and energy. Carbon and nitrogen are two important materials for cell construction. In the multiple substrate, both carbohydrate and protein supply carbon for cell growth, but only protein supplies nitrogen. In the non-peptone containing experiment (glucose/peptone = 5/0), peptone was replaced with ammonia as the nitrogen source. However, ammonia could just provide one nitrogen source, unlike peptone which was so advantageous for hydrogen fermentation. The cell growth in the experiment with substrates containing 40% peptone was almost three times that using glucose as the sole substrate. A similar result was obtained in the study of Ueno in 2001. In Ueno's study, the cell growth and hydrogen production in the experiment using peptone plus ammonia as nitrogen source was twice that in the experiment using ammonia as the sole nitrogen source.

Glucose could provide the necessary carbon material for cell growth and be fermented to produce significant energy for growth, biosynthesis and other cell activities. Substrate energy increased with glucose contained in the substrates. To integrate material and energy demand, the best substrate design for hydrogen producing bacteria was 60% glucose and 40% peptone.

In our previous study (Bai *et al.*, 2001), it was ascertained that the protein substrate was inferior to carbohydrate for hydrogen production. However, in this work, the protein contained in the substrate was not useless. Protein could improve the cell growth of hydrogen producing bacteria and consequently increase hydrogen production. Brosseau *et al.* (1986) observed that hydrogen production and cell growth occurred simultaneously. If the environmental condition was not suitable for cell growth, the fermentation would follow other pathways, but not the hydrogen-producing pathway, even if the substrates were suitable for hydrogen fermentation such as the fermentation using the carbohydrate as sole substrate. The multiple substrates containing 60% glucose and 40% peptone provide good conditions not only for cell growth, but also for hydrogen production. The hydrogen production in the experiment involving substrates containing 40% of peptone was approximately 4.5 times greater than that using glucose as the sole substrate.

To clarify the advantage of protein for cell growth and hydrogen production, this study compared two nitrogen sources, peptone and ammonia. Figure 2 illustrates the results of hydrogen fermentation in the serial transfer culture with protein and ammonia,

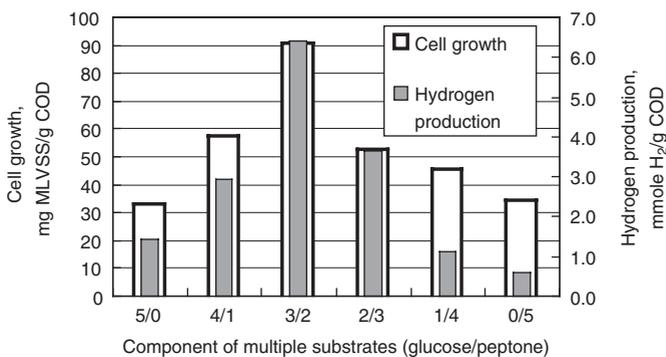


Figure 1 Cell growth and hydrogen production in the fermentation of sole substrates, glucose (5/0) and peptone (0/5), and multiple substrates, with various glucose and peptone contents (glucose/peptone = 4/1, 3/2, 2/3, 1/4). All experiments had the same total quantity of substrates, 5,000 mg-COD/L

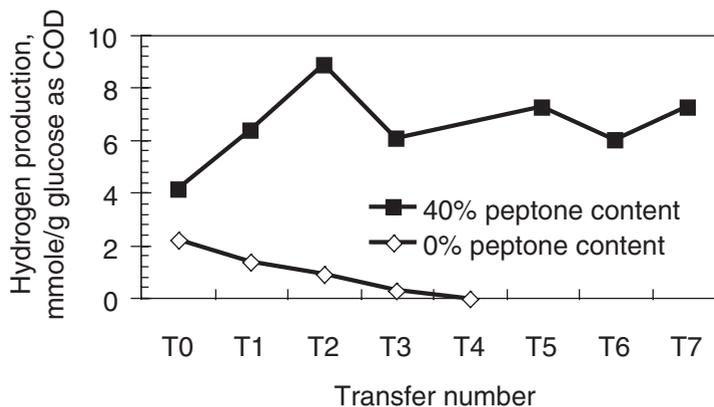


Figure 2 Comparison of the hydrogen yields in the fermentation using peptone and ammonia as the sole nitrogen source

respectively, as the nitrogen source. In the transfer cultures using ammonia as the sole nitrogen source, the cell growth and hydrogen production decreased steadily following the transfers. Moreover, the hydrogen production was stopped following four transfer cultures. However, the hydrogen production could be maintained in the range from 6 to 8 mmole H_2/g glucose as COD in the transfer cultures with peptone as the sole nitrogen source.

By investigating the bacteria morphology and by analyzing the 16SrDNA, the dominative micro flora in the mixed culture used in this work was identified as *Clostridium* (data not shown). This genus was commonly reported to require specific nutrients for growth, such as biotin, folic acid and so on (Bergey's Manual of Systematic Bacteriology, 1989). Although these required nutrients were intentionally added to the broth for cell growth, the protein substrates may also contain some unknown necessary nutrients for growth, such as amino acids. Ammonia simply provides the nitrogen for cell growth, but not the required amino acids. Following the culture transfer, the necessary nutrients decrease and hydrogen production and cell growth worsen without protein addition to substrate. Therefore, most previous studies on obtaining high hydrogen production have used media containing peptone, polypepton or yeast extract to enhance the growth and activity of hydrogen-producing bacteria. (Taguchi *et al.*, 1996; Yokoi *et al.*, 1995; Ueno *et al.*, 2001; Majizat *et al.*, 1997).

That ammonia could not be substituted for protein was suggested according to the results presented in this investigation. Furthermore, the importance of the protein was verified again by transferring the T3 of the serial experiments involving ammonia as the only nitrogen source to the new broth containing 40% peptone. The cell growth, hydrogen production and fatty acid accumulation could increase the levels of the serial experiments with substrates containing 40% peptone. These results confirmed the importance of protein and demonstrated that the hypothesis clearly indicated that protein substrates contained some necessary nutrients useful for the cell growth and hydrogen production.

Alkalinity provided by protein fermentative products

In the fermentation of multiple substrates, the greater ratio of peptone contained in the substrates caused the higher final pH value. The substrates containing less than 40% peptone caused the final pH to be below 5. Low pH value advanced the scheduled time of the bioreaction stoppage in batch fermentation. Therefore, the glucose conversion ratios of the substrates containing only 0% and 20% peptone were below 80%. However, the glucose conversion ratios of the substrates containing 40%, 60% and 80% peptone were all exceeding 95% (Figure 3).

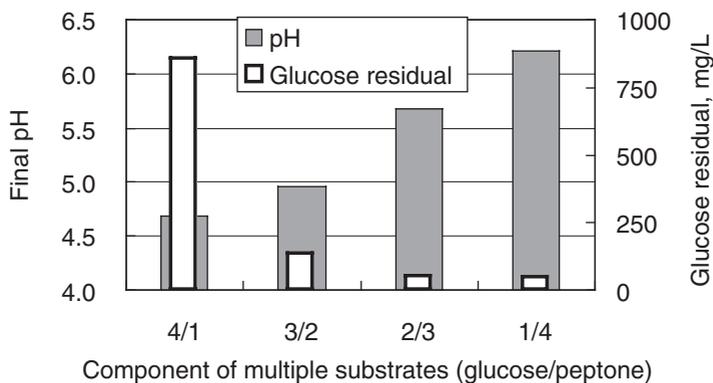


Figure 3 Final pH and glucose residual following the hydrogen fermentation of multiple substrates with various ratios of glucose to peptone (4/1, 3/2, 2/3, 1/4). All experiments had the same total quantity of substrates, 5,000 mg-COD/L

was ammonia. Ammonia and amino group could provide alkalinity to maintain suitable pH for hydrogen fermentation. However, hydrogen fermentation was followed by the production of various organic acids, such as formic acid, acetic acid and butyl acid and so on that would cause serious acidification, and would create an environment unsuitable for cell growth and hydrogen fermentation. Consequently, protein contained in multiple substrates could repress the acidification in hydrogen fermentation and avoid the phenomenon of low pH limiting cell growth and hydrogen production.

In this study, the optimal hydrogen fermentation was achieved using the multiple substrates containing 40% peptone, and had a final pH value of approximately 5. Ginkel *et al.* (2001) determined that the highest hydrogen production rate occurred at pH 5.5. Moreover, Fang and Liu (2002) also observed that the optimal pH was located at 5.5 in the fermentation of glucose by mixed culture. Fang and Liu (2002) also pointed out that the highest residue of glucose occurred under low pH condition (< 5) and the methane appeared under high pH condition (> 6). In this investigation, the phenomenon of low peptone content causing final low pH, which in turn inhibits cell growth, hydrogen production and glucose utilization, was similar to that introduced by Fang and Liu (2002). However, the reason for the low hydrogen production caused by the high peptone containing substrate was different to high pH. This study did not observe methane in any experiments because of the high quality control on sterilization. The multiple substrates containing more peptone could provide more alkalinity, and the final pH of the fermentation was also higher. However, hydrogen production could not be improved further when peptone content exceeded 40%, because peptone was not a good electron donor for hydrogen production.

Fermentative products in the fermentation of multiple substrates

In our early study (Bai *et al.*, 2001), analysis of fermentative products verified that acetate was the common product in the fermentations of carbohydrate and protein. The acetate yield in the fermentation of peptone significantly exceeded that of glucose. Therefore, the accumulation of acetate increased with peptone content in multiple substrates. In carbohydrate biodegradation, acetate and butyrate were two byproducts in the hydrogen producing pathway (Jones and Woods, 1986). The production of acetate and butyrate depends on that of hydrogen. However, acetate is also the main product of the fermentation of peptone. Therefore, butyrate is the only byproduct whose accumulation depends on hydrogen production due to glucose degradation. As in cell growth and hydrogen production, the maximum accumulation of butyrate, 1.7 mmole/g substrate as COD, occurred in

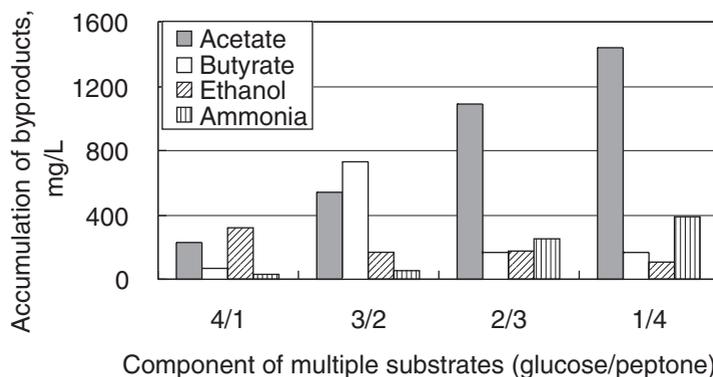


Figure 4 Byproducts of the hydrogen fermentation of multiple substrates with various ratios of glucose to peptone (4/1, 3/2, 2/3, 1/4). All experiments had the same total quantity of substrates, 5,000 mg-COD/L

the fermentation of multiple substrates containing 3,000 mg-COD/L of glucose and 2,000 mg-COD/L of peptone (Figure 4).

Acetate and ammonia accumulations both increased following increased peptone content in multiple substrates. Acetate was a mutual product of fermentation of both glucose and peptone, but ammonia was a product only in the fermentation of peptone. The change of ammonia accumulation in each experiment is used to elucidate the extent of fermented peptone. In the fermentation of multiple substrates containing 3,000 mg-COD/L of glucose and 2,000 mg-COD/L of peptone, only about 20% of the organic nitrogen contained in peptone was converted to ammonia. The conversion ratio of organic nitrogen to biomass thus was estimated to be approximately 19%. The other 61% of nitrogen remained of organic type. Peptone was not utilized completely. On the other hand, about 69% organic nitrogen was degraded to ammonia in the fermentation using 1,000 mg-COD/L of glucose and 4,000 mg-COD/L of peptone as substrate. A greater peptone fraction in substrate increased the accumulation of acetate but did not increase hydrogen production.

Therefore, in the fermentation of multiple substrates with high hydrogen production efficiency, carbohydrates share most responsibility for donating electrons for hydrogen production, and proteins share responsibility for providing the necessary factors for cell growth. Protein should avoid being utilized for fermentation. Fermentation of protein causes the production of fatty acids and ammonia which may inhibit hydrogen production, even if the concentration required to cause such inhibition is very high (Colin *et al.*, 2001; Sterling *et al.*, 2001). Moreover, protein fermentation could cause the hydrogen consumption bioreaction that seriously depressed hydrogen recovery (Bai *et al.*, 2001).

Fermentation of multiple substrates containing starch and peptone

Starch, a more complex carbohydrate than glucose, was used in hydrogen fermentation instead of glucose. The amount of cell growth varied according to the change of substrate composition, similar to the behavior observed in the experiment with glucose as the carbohydrate substrates stated previously. The optimal composition of substrates for cell growth was 3,000 mg-COD/L of starch and 2,000 mg-COD/L of peptone, and could be fermented to achieve a yield of 146 mg-MLVSS/g-COD (Figure 5).

The fermentation using a multiple substrate containing 4,000 mg-COD/L of starch and 1,000-mg COD/L of peptone demonstrated maximum hydrogen production. This behavior differs from the fermentation containing glucose. Starch is a more complex carbohydrate and demonstrates a slower degradation rate than glucose. Comparing the fermentation of different multiple substrates, peptone demonstrated a greater edge when competing with

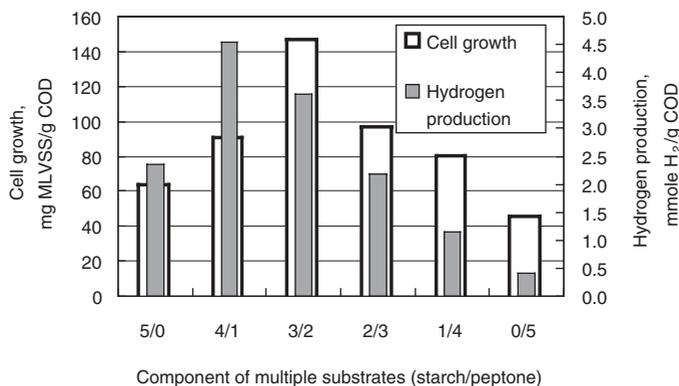


Figure 5 Cell growth and hydrogen production in the fermentation of sole substrates, starch (5/0) and peptone (0/5), and multiple substrates with various starch and peptone contents (starch/peptone = 4/1, 3/2, 2/3, 1/4). All experiments involved equal numbers of overall substrates, 5,000 mg-COD/L

starch than when competing with glucose. The effects of peptone, such as providing alkalinity to maintain suitable pH and providing necessary nutrients to improve cell growth, may be enforced, because peptone degraded earlier in the batch fermentation containing starch than in that containing glucose. On the other hand, the negative effects, such as hydrogen consumption, may be repressed because the hydrogen production by fermenting starch occurred after peptone fermentation. Therefore, the hydrogen production of multiple substrates containing starch exceeded that of multiple substrates containing glucose when peptone content exceeded 40%.

Conclusions

This study investigated the effects of substrate composition on hydrogen fermentation of multiple substrates. The peptone content in the substrate directly influenced cell growth, final pH value, production of acetate, and ammonia. By these influencing parameters, peptone could indirectly influence hydrogen production. The optimal substrates for hydrogen production contained 3,000 mg-COD/L of glucose and 2,000 mg-COD/L of peptone or 4,000 mg-COD/L of starch and 1,000 mg-COD/L of peptone. By fermenting the optimal multiple substrates, carbohydrate endeavors to provide electrons for hydrogen production, but protein merely provides the nutrient for improving cell growth and maintaining suitable pH condition for hydrogen fermentation.

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