Enrichment and adaptation of extreme-thermophilic (70°C) H₂ producing bacteria to organic household solid waste by repeated batch cultivations

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Abstract Adaptation of biohydrogen producing extreme-thermophilic bacteria to household solid waste (HSW) was investigated. Inoculum received from a extreme-thermophilic glucose fermentation reactor was exposed to increasing HSW concentrations from 1 g VS/L to 10 g VS/L via repeated batch cultivations. It was found that repeated batch cultivation was a very useful method to adapt and enrich biohydrogen producing cultures that could ferment HSW with high hydrogen yield and without significant lag phase. For unadapted cultures (inocula from simple substrate- glucose to complex substrate-HSW), hydrogen was produced only in the HSW concentration of 1-2 gVS/L and the lag phase required more than 2 days. After adaptation, hydrogen was produced directly in the HSW feedstock (10 gVS/L) with the maximum yield of 101.7±9.1 mL H₂/gVS added. Acetic acid was the main fermentation product in all HSW concentration cultivations.

Key words biohydrogen; household solid waste; repeated batch cultivation; extreme-thermophilic

Introduction
Hydrogen gas has been deemed the fuel of the future, and it is believed that a hydrogen fuel based economy would be more environmentally friendly than the one of fossil fuels. Among the hydrogen production methods, the most promising and environmentally friendly method seems to be dark fermentation from organic wastes as it combines the hydrogen generation with waste treatment (Benemann 1996). Hydrogen producing bacteria can grow in a wide temperature range from ambient to extreme-thermophilic. Research at extreme-thermophilic temperatures gains increasing interest because the hydrogen production yield is much higher than the ones in mesophilic and thermophilic temperatures (Reith et al. 2003). In addition, extreme-thermophilic conditions result in higher hydrolysis activity, which is the bottleneck for degradation of complex substrates such as manure and household solid waste (Hartmann and Ahring 2005). Moreover, extreme-thermophilic conditions have the advantage of better sanitation and lesser contamination chance from methanogens (Kotsopoulos et al. 2006).

Up to now, studies concerning extreme-thermophilic hydrogen fermentation have mainly been focused on pure cultures isolated from extreme environment, such as deep-sea volcanoes and hot springs. However, for a technologically feasible process, stable, mixed cultures easily obtainable from natural sources able to operate on non-sterile feedstock are required (Hawkes et al. 2002). In most cases the mixed culture inocula need to be enriched and adapted from inocula obtained from thermophilic environments, as extreme-thermophilic inocula are often not available. In the previous study of HSW fermentation, we used thermophilic manure inocula to cultivate extreme-thermophilic bacteria directly in a continuously stirred tank reactor, and found that its H₂ production yield in steady state period was lower than the one in mesophilic conditions (Liu et al. submitted). It suggests that the enrichment way was not successful. Repeated batch cultivation is a well-known method for enhancing the productivity of microbial cultures through extending the production phase of the culture by replacing a portion of the original culture with fresh substrate. Weigand (1981) reported that the repeated batch cultivation obtained the highest productivity increase comparing to fed batch and continuous cultivation methods. Furthermore, repeated batch cultivation has operational advantages, such as avoiding variation in the inoculum and maintaining the microorganism at high growth rates (Fabregas et al. 1996). The aim of this study is to investigate the
H₂ production capability during adaptation and cultivation of hydrogen producing mixed cultures for HSW fermentation under extreme-thermophilic condition via repeated batch cultivation.

Materials And Methods
Inoculum and feedstock
The initial inocula were taken from hydrogen fermentation reactors using glucose and synthetic BA media under 70°C (Kotsopoulos et al., 2006). The household solid waste came from a biogas plant in Grindsted, Denmark (Liu et al. 2006). Total solid (TS) of raw HSW was 35.1±2.1% (w/w) and 75% of the TS was volatile. To prepare the feedstock for the experiments, raw HSW was shredded in a mechanical meat mincer, and then screened through a 0.7 mm diameter sieve to avoid blocking of the pump. Finally the screened HSW was diluted with tap water to volatile solid (VS) of 2.0±0.16% and used as the feedstock.

Repeated batch cultivation
Batch experiments were carried out under hyper thermophilic conditions (70°C) in 65 mL sealed serum vials, with working volume of 20 mL, under an atmosphere of N₂-CO₂ (80/20, vol/vol) without shaking. In order to evaluate the effect of shifting the substrate from glucose to HSW, batch experiments with HSW concentration of 1-5 gVS/L were performed. The ratio of HSW: BA medium: Inoculum is shown in Table 1. Each HSW concentration was tested in triplicate.

<table>
<thead>
<tr>
<th>HSW</th>
<th>Unit</th>
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<tbody>
<tr>
<td>1 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>2 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>3 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>4 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>5 gVS/L</td>
<td>mL</td>
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<tr>
<td>6 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>7 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>8 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>9 gVS/L</td>
<td>mL</td>
</tr>
<tr>
<td>10 gVS/L</td>
<td>mL</td>
</tr>
</tbody>
</table>

For repeated batch cultivation, mixed cultures from the first generation having the highest H₂ production were used as the inoculum of the second generation. Repeated transfer was stopped when no hydrogen yield increase compared to the previous cultivation was observed. The repeated batch cultivation started from HSW concentration of 1gVS/L and then increased to 2, 3, 4, 6 and 10gVS/L gradually. No pH adjustment was applied. Each HSW concentration experiment was carried out in 12 replicates.

Analysis
Total solid, volatile solid were determined according to the procedures described in Standard Methods (APHA 1995). The percentages of hydrogen, methane, CO₂ in the gas phase were determined using a gas chromatograph equipped with a thermal conductivity detector. Volatile fatty acids (VFAs) and ethanol were determined by a GC with a flame ionization detector while lactate was measured by HPLC.

Results
Effect of substrate change from glucose to HSW
In the first generation when substrate was changed from glucose to different concentration of HSW,
hydrogen was not detected when HSW was from 3 to 5 g/L (Figure 1). For 1 g/L HSW, the hydrogen production started at day 2 and reached the maximum hydrogen production yield of 84.3±2.7 mL H₂/gVS added at day 16. For 2 g/L HSW, hydrogen was generated starting at day 5 and a maximum production of 23.5±13.1 mL H₂/gVS added was achieved.

**Figure 1.** Profiles of H₂ yields in the 1st generation of HSW cultivation at concentration of 1 to 5 gVS/L

**Figure 2.** Hydrogen production profiles of 1 gHSW-VS/L cultivation in consecutive 5 generations

**Repeated Batch Cultivation**

Figure 2 shows hydrogen production yields of 1 gHSW-VS/L cultivation in consecutive 5 generations. Hydrogen was produced faster and higher with the transfer of mixed cultures. In the first generation, it took 15 days to reach the maximum hydrogen production yield of 84.3±2.7 mL H₂/gVS added. During the second generation, after 8 days, the hydrogen production was stable at 106.2±5.4 mL H₂/gVS added. In the third generation and the fourth generation, the hydrogen production stabilized after 6 and 4 days, the
maximum hydrogen production was 122.7±14.1 and 157.3±21.1 mL H₂/g VS added, respectively. In the fifth generation, the hydrogen production started after 4 hours and increased to 106.4±26.3 mL H₂/g VS added after 24 hours. After 4 days, the hydrogen production achieved the maximum, which was 169.5±11.8 mL H₂/g VS added. After five consecutive transfers, the H₂ production profile was very similar (data not shown), then the same approach of repeated batch cultivation was conducted in 2-10 g HSW-VS/L concentrations.

The adaptation of 2 to 10 g HSW-VS/L had the same trend as 1 g VS/L. The hydrogen production was higher and faster with the transfers. Figure 3 illustrates the hydrogen production profiles of the final generation in 2-10 g VS/L cultivation. It can be seen that after repeated batch cultivation, the maximum hydrogen production was achieved after 4-5 days in most of HSW concentrations. The final acetate, hydrogen yields and pH of each HSW concentration in Figure 3 are summarized in Table 2. Hydrogen production yields dropped from 170 mL H₂/g VS added in 1 g VS/L to 125 mL H₂/g VS added in 2 g VS/L and then stayed at 100 mL H₂/g VS added in 3-10 g VS/L.

**Figure 3.** H₂ production yields in the final generation of repeated batch cultivation in 2 to 10 g VS/L.

Acetate was the main product detected, covering more than 90% of total VFA for all HSW concentrations tested. Lactate, ethanol, butanol were detected in small amounts. Acetate concentration increased from 14 mM in 1 g/L to 90 mM in batches with 10 g/L HSW, however, the yields in all HSW concentrations were similar and were around 8.5 mmol acetate/g VS added. pH decreased during the cultivation as acetate production. The initial pH in all HSW concentration cultivation were dropping with the increase of HSW concentration (7.26 in 1 g/L to 5.5 in 10 g/L). This is likely caused by the fact that less BA medium (containing bicarbonate buffer) and more HSW were added with the increase of HSW concentration. In 10 g VS/L cultivation, no BA medium was required. pH started from 5.5 which was the feedstock pH and decreased to 4.45 at the end. It is worth to mention that during 10 g/L cultivation, it took more than 15 repeated transfers to achieve the stable hydrogen production.
Table 2. Summary of acetate and \( \text{H}_2 \) yields and pH in the final generation of 1 to 10gVS/L in Figure 3

<table>
<thead>
<tr>
<th>HSW gVS/L</th>
<th>( \text{H}_2 ) mL/gVS\text{added}</th>
<th>Acetate mM</th>
<th>Acetate mmol/gVS\text{added}</th>
<th>pH starting</th>
<th>pH end</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>169.5±11.8</td>
<td>9.39±1.59</td>
<td>9.39±1.59</td>
<td>7.26±0.02</td>
<td>6.64±0.16</td>
</tr>
<tr>
<td>2</td>
<td>125.1±13.1</td>
<td>20.41±3.37</td>
<td>10.21±1.69</td>
<td>7.18±0.08</td>
<td>6.02±0.14</td>
</tr>
<tr>
<td>3</td>
<td>108.2±14.8</td>
<td>27.17±3.94</td>
<td>9.06±1.31</td>
<td>6.92±0.10</td>
<td>5.29±0.11</td>
</tr>
<tr>
<td>4</td>
<td>104.3±11.3</td>
<td>37.24±5.18</td>
<td>9.31±1.29</td>
<td>6.83±0.07</td>
<td>5.17±0.09</td>
</tr>
<tr>
<td>6</td>
<td>105.2±5.9</td>
<td>59.02±11.72</td>
<td>9.83±1.95</td>
<td>6.40±0.14</td>
<td>4.97±0.12</td>
</tr>
<tr>
<td>10</td>
<td>101.7±9.1</td>
<td>89.83±9.01</td>
<td>8.98±0.91</td>
<td>5.5±0.15</td>
<td>4.45±0.10</td>
</tr>
</tbody>
</table>

Discussion

This study demonstrates that the method of repeated batch cultivation is very useful to cultivate and adapt the hydrogen producing cultures from simple substrate (glucose) to complex substrate like HSW. Without adaptation, no hydrogen was produced when HSW concentration was over 2 gVS/L. Furthermore, it took 2 days to start hydrogen production in 1 gVS/L and the maximum hydrogen yield was 84 mL/gVSS\text{added}. However, after adaptation via repeated batch cultivation, hydrogen could be produced directly in the HSW feedstock without any dilution (10 gVS/L). The lag phase was reduced to a couple of hours and the hydrogen yield was double (169 vs 84 mL/gVSS\text{added}) in 1 gVS/L. It shall be noted that repeated batch cultivation was time consuming. The total adaptation and cultivation from 1g HSW to 10g HSW in this study lasted 9 months. For each HSW concentration, 5 to 10 transfers were needed to achieve the maximum hydrogen production and it normally lasted at least 1 month. It has been claimed by other researchers that the repeated batch cultivation needs a long time to make the bacteria adapted, for example, Imachi (2000) has reported a successful propionate oxidizing bacteria cultivation by repeated transfer more than 10 times lasted 20 months.

The long adaptation period suggests inhibition was happening in this study. It is obvious that substrate inhibition plays a big role especially in the beginning of adaptation (such as from glucose to HSW). Product inhibition shall be considered too. Under extreme-thermophilic temperature, degradation of complex organic substrate like HSW may generate some toxic compounds which could be harmful to microorganisms. Even the simple product, like acetate, has been reported to inhibit hydrogen production. Van Niel et al. (2003) found that hydrogen production was decreased along with the acetate concentration increase from 0-400mM. However, some studies found that acetate inhibition could be overcome by repeated batch cultivation (Arnold et al. 2002; Nakashimada et al. 1999). It indicates that the method of repeated batch cultivation would enhance the selection of right microorganisms. Our microbiological analysis via DGGE (data not shown) supported this hypothesis as the dominated microorganisms were changed during the cultivation period from 1 to 10 gVS/L experiments.

The VFA results show that acetic acid fermentation pathway was the main pathway in repeated batch cultivation. Acetic acid pathway corresponds to the maximum hydrogen yield of 4 moles hydrogen per mole glucose. Comparing to our former research which used unadapted culture as inoculum, lactate pathway covered 50% of the total fermentation pathways (Dawei et al. submitted), resulting in the low hydrogen yield of 11 mL \( \text{H}_2/g\text{VSS}\text{added} \). It is well known when lactate was the fermentation product, no hydrogen was produced. It suggests that repeated batch cultivation could adapt and cultivate the cultures to lead the fermentation pathway to favorable hydrogen production.
Conclusions

Extreme-thermophilic hydrogen production from household solid waste was investigated via repeated batch cultivations from the concentrations of 1 g VS/L to 10 g VS/L. It was found that repeated batch cultivation was a very useful method to adapt and cultivate the cultures to enhance the hydrogen production and reduce the lag phase. Without adaptation, hydrogen was produced only in the HSW concentration of 1-2 g/L and the lag phase required more than 2 days. After adaptation, hydrogen was produced directly in the HSW feedstock (10 gVS/L) with the maximum yield of 101.7±9.1 mL H₂/gVS added. The lag phase was reduced to couples of hours. Acetic acid was the main fermentation product in all HSW concentration cultivations.

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Reference


