**Biological Hydrogen Production: Simultaneous Saccharification And Fermentation With Nitrogen And Phosphorus Removal From Wastewater Effluent**

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Biological hydrogen production: Simultaneous saccharification and fermentation with nitrogen and phosphorus removal from wastewater effluent

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ABSTRACT

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1. Introduction

1.1. Hydrogen production

Hydrogen is potentially an ideal fuel since its only oxidation product is water. When used in a fuel cell to generate electricity, it is up to three times as efficient as an internal combustion engine [1]. However, its production, primarily from steam reformation of natural gas at 700–1100 °C, is energy-intensive and completely dependent on fossil fuel (Eq. (1)).

\[ \text{CH}_4 + \text{H}_2\text{O} \rightarrow \text{CO} + 3\text{H}_2 \] (1)

Hydrogen can also be produced by electrolysis, splitting water into its component gases, hydrogen and oxygen (Eq. (2)), although the electrical demand, and therefore cost, is high.

\[ 2\text{H}_2\text{O}(l) \rightarrow 2\text{H}_2(g) + \text{O}_2(g); \quad E_0 = +1.229 \text{ V} \] (2)

Biological hydrogen production, typically using anaerobic bacteria or photosynthetic algae, occurs catalytically at ambient temperature and pressure. Because hydrogen has little solubility in water (<0.0015 g H₂ per kg water at 30 °C) [2], it quickly accumulates in the headspace of the reactor where it can be easily collected. If developed into a stable and economically viable process, it might provide a means to produce useful amounts of hydrogen from renewable or discarded materials.

Bacteria can catalyze the production of hydrogen with either hydrogenase or nitrogenase enzymes [3, for review]. Recent research on hydrogenase enzymes has been reviewed by English et al. [4]. The enzyme catalyzes the reversible...
oxidation of molecular hydrogen, and the reaction can be most simply written as:

\[
H_2 \rightarrow 2H^+ + 2e^- \quad (3)
\]

1.2. Nitrogen fixation

Nitrogenases enzymes catalyze the reduction of atmospheric nitrogen (N\(_2\)) to ammonia and are found only in nitrogen-fixing bacteria. They are typically down-regulated by the presence of ammonia, to avoid the energetically expensive fixation of nitrogen when not needed by the cell. Molybdenum-containing nitrogenases, the most common type found, catalyze the production of hydrogen in addition to ammonia at the rate of 1 mol of H\(_2\) per mole of N\(_2\) fixed [5]:

\[
N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (4)
\]

Relatively few (perhaps 100) bacteria possess this capability, which is critical in nature because the supply of fixed nitrogen to the biosphere is rate-limiting for biological activity in most areas of the planet. Both nucleic acids and proteins require nitrogen for their biosynthesis. Bacteria of the Azotobacter genus are one example frequently used as model organisms in fermentation studies [6]. Biological nitrogen fixation provides about 40% of the nitrogen found in the world’s soil and water [5].

Industrially, nitrogen fixation is typically accomplished using the Haber–Bosch process, in which hydrogen is first produced from methane (Eq. (1)), then ammonia is produced from hydrogen and nitrogen (Eq. (5)).

\[
N_2(g) + 3H_2(g) \rightarrow 2NH_3(g) \quad (5)
\]

Agronomists have calculated that well over 1/3 of the world’s present population is fed by virtue of the Haber–Bosch process [7]. The reaction is of great economic importance since the world’s industrial production of nitrogenous fertilizer increased 27-fold between 1950 and 1990, when it reached 8 x 10\(^7\) tonnes N/year [8]. Currently, 1% of the world’s energy supplies are consumed in the industrial fixation of nitrogen through the Haber–Bosch process [9], leading to a potential confluence of energy and fertilizer crises.

Biological nitrogen fixation provides a catalytic alternative to the commercial fixation of nitrogen, and its broader use could help decouple the price of fertilizer from the price of natural gas. A nitrogen-fixing, hydrogen-producing culture offers the potential to simultaneously produce both organic ammonia and hydrogen from renewable materials. Used in conjunction with a relatively carbon-rich material such as paper, it could also be useful for the removal of nitrogen and/or phosphorus from wastewater effluent to prevent eutrophication in receiving waters.

1.3. Biological nitrogen and phosphorus removal

The Chesapeake Bay in the Eastern United States is an example of a body of water suffering from a high load of nitrogen and phosphorus. U.S. Executive Order 13508 “Chesapeake Bay Protection and Restoration”, dated May 12, 2009, describes action necessary to respond to a pollution crisis affecting the Chesapeake Bay. The order states “The main contaminants affecting the Bay are nitrogen, phosphorus and sediment. In 2008, the estimated loads of contaminants from the Bay watershed included 311 million pounds of nitrogen and 19 million pounds of phosphorus... EPA (Environmental Protection Agency) estimates that in order to achieve water quality standards for the Bay, the nitrogen load must be reduced by 44% and the phosphorus loading cut by 27.5%.”. The target date for these goals is 2025. This effort will be administered by a Federal Leadership Committee which includes the Department of Defense.

In this study we sought to determine the efficiency with which shredded paper could be biologically converted to hydrogen using the discharge effluent from the Aberdeen Proving Ground (APG) Edgewood wastewater treatment plant (WWTP) as the medium and the sole source of micronutrients and organisms. This plant treats low-strength waste consisting almost totally of human waste with essentially no industrial waste component. It discharges to a tributary of the Chesapeake Bay. Simultaneously, we sought to determine the efficiency with which nitrogen and phosphorus could be removed from the WWTP discharge waters using the same biological process as used to generate hydrogen.

Two reactor configurations were used; a sequencing batch reactor (SBR) and a classic batch reactor (CBR). Paper, in addition to being carbon-rich, has the added advantage that it can be directly catalyzed to monosaccharides by cellulases without requiring thermochemical pretreatment, as would typically be required with lignocellulosic feedstocks. Therefore it offers a readily-processed and reproducible substrate with which to test various reactor conditions and configurations, data from which may inform similar processes conducted with higher impact feedstocks such as corn stover or switchgrass.

1.4. Batch reactor configurations

The SBR is a periodically operated reactor, frequently used in wastewater treatment operations. It offers the simplicity and control of a batch reactor with the kinetic advantages of a plug flow reactor followed by a continuously stirred tank reactor. Organisms are settled (concentrated) and retained after each cycle. The CBR is a tank that is filled, stirred for the duration of the cycle, and completely drained at the end. It typically offers greater simplicity of operation but does not concentrate the organisms between cycles.

2. Materials and methods

2.1. Materials

Post-treatment wastewater effluent was collected from the discharge area at the APG Edgewood treatment plant and used as the sole source of organisms, medium, and nutrients (other than paper) in the bioreactors. Specifically, the organisms used were only those naturally occurring in the wastewater. Neither were any other inorganic nutrients added; the experiments utilized only those found naturally in the wastewater effluent, including nitrogen and phosphorus compounds which were measured and reported for each experiment performed.

Paper used was SkilCraft (Lab Shreveport, LA) Recycled Copy Paper 7530-01-334-7817 Recycled (30% PCW), and comprised the only exogenously-added organic nutrient.
2.2. **Equipment**

Bioreactor studies were conducted in New Brunswick BioFlo 110 5 L vessels. Nitrogen (5 ml/min) was sparged through the bioreactor to maintain anaerobicity. Hydrogen detection was accomplished with a HY-OPTIMA™ 700 in-line process hydrogen monitor (H2scan, Valencia, CA), sealed in a separate vessel connected to the reactor headspace. The monitor was factory-calibrated with 0.5–100% hydrogen standards traceable to the National Institute of Standards and Technology. The pH of the reactors was controlled throughout all fermentations by the automatic addition of 0.1 N NaOH. Total organic carbon (TOC), ammonia nitrogen, nitrate nitrogen, nitrite nitrogen and phosphorus analyses were conducted with the respective kits from Hach (Loveland, CO).

2.3. **SBR and CBR methods**

The SBR was established using WWTP effluent and shredded paper, as described in Materials and Methods, above. The reactor was initially seeded with effluent, collected and analyzed in batch, and was fed paper and cellulase on a periodic basis, 2–4 times per week as appropriate until the mixed liquor suspended solids (MLSS) levels were above 3000 mg/L. Initial batches of wastepaper feed raised the pH and required adjustment with HCl to pH 5.0. However, as the reactor equilibrated, an effective buffer was established (presumably due to the organic acid products of paper biodegradation) and pH changed very little upon paper addition. Once the reactor MLSS was established above 3000 mg/L, paper was added in 20 g batches along with 9 ml of Accellerase 1500 enzyme solution (a kind gift from Genencor International). Conditions were those of simultaneous saccharification and fermentation (SSF, meaning that enzymatic degradation and fermentation were conducted simultaneously in the same vessel). Fermentation of the Accellerase 1500 solution alone (without paper) produced no detectable hydrogen when tested at the maximum concentration used and under optimal conditions determined for hydrogen production.

The CBR was operated on a periodic basis by filling the reactor (5L) with fresh effluent (used within three days of collection), adding 20 g of paper and nine ml of Accellerase 1500 enzyme (also SSF conditions), reacting for several days until hydrogen production was complete, draining the reactor completely, and repeating the same cycle.

Cellulase concentrations were based on the manufacturer’s recommendations (0.15–0.45 ml enzyme solution per gram of substrate). In our studies, 0.45 ml/g gave the best results, yielding almost three times more hydrogen than 0.15 ml/g. Cultures using shredded paper alone with no enzyme produced very little hydrogen.

2.4. **Nuclear magnetic resonance (NMR) methods**

Analysis of solution-state reaction products was carried out at 25 °C using Bruker AVANCE DRX-300 MHz and a Bruker DRX-500 MHz NMR spectrometers. The DRX-300 MHz was equipped with a QNP probe, while the DRX-500 MHz was equipped with a cryogenic TCI probe with enhanced detection of 1H and 13C. The NMR experiments performed were as follows: 1H zg with and without solvent presaturation pulse, 13C attached proton test (Bruker jmod pulse program), 1H–13C Heteronuclear Multiple Quantum Coherence, 13C–[1H] zgdc.

3. **Theory/calculation**

The central hypothesis of this work was that it would be possible to run an anaerobic bioreactor using only shredded paper, commercially-available cellulase enzymes, and wastewater treatment plant effluent to simultaneously produce significant amounts of hydrogen while removing nitrogen and phosphorus from the effluent.

Assuming paper to be comprised of 100% cellulose, its molecular formula would be (C6H10O5)n, corresponding to 44% carbon by weight:

\[(12.01g/molC + 6)/[(12.01g/molC + 6) + (1.01g/molH + 10) + (16.00g/molO + 5)] \times 100\]

= 44.44% C

This would yield 888 lbs C per ton of paper:

0.4444 lb C/lb paper \times 2000 lb/1 ton \rightarrow 888 lb C per ton of paper

Assuming a carbon:nitrogen:phosphorus biological demand ratio of 100:10:1, biodegradation of one ton of paper could remove 88 lbs of nitrogen and 8.8 lbs of phosphorus that would otherwise be discharged to receiving waters. As a practical example, a WWTP discharging one million gallons per day of effluent containing 10 mg/L nitrogen (83 lbs nitrogen per day) would require 0.85 tonnes of paper per day to remove about 90% of its nitrogen from the effluent, and partition that nitrogen to the solid phase where it could be separated with typical solids handling equipment and recycled as fertilizer.

4. **Results**

4.1. **Reactor setup and operation**

The SBR was initially established as described in Section 2.3 above, and was operated for a total of 100 days. Hypothetically, the concentration of organisms resulting from the repeated settling of the reactor could lead to a more efficient process for hydrogen production. However, since biological...
hydrogen production in mixed cultures is in equilibrium with biological hydrogen consumption (often by methanogens), it was also possible that overall hydrogen yields would decrease.

The WWTP effluent that was used as influent to the SBR was analyzed for TOC, ammonia, nitrate, nitrite and phosphorus concentrations. The SBR effluent was periodically analyzed for MLSS, effluent suspended solids (ESS), TOC, ammonia, nitrate, nitrite, phosphorus, and hydrogen production. Fig. 1 shows the MLSS on the basis of individual measurements and calculated as a 10 day moving average. Solids concentrations ranged between about 3000 and 9000 mg/L. Effluent TOC averaged 856 mg/L and ESS averaged 266 mg/L.

CBR operation was much simpler since it involved no concentration of biomass via settling. The tank was simply filled with WWTP effluent to which paper and enzyme were added. When hydrogen production was complete, the tank was drained and re-filled.

4.2 Operational comparison: SBR and CBR

The reactors were compared with regard to hydrogen production, nitrogen and phosphorus removal, and final products, as detected by NMR. Initial hydrogen production levels were similar in the two reactors but declined over time as MLSS increased in the SBR. The CBR however, maintained initial levels over the course of repeated batch operations (Fig. 2).

Total nitrogen (nitrate + nitrite + ammonia) and phosphorus concentrations were determined for the influent and periodically from the effluent of both reactors (Table 1). The SBR performed slightly better in terms of its efficiency of total nitrogen removal (95% vs. 92%) and significantly better in terms of its efficiency of phosphorus removal (97% vs. 56%). Part of the difference in efficiency was attributable to the higher starting values for the SBR feed, especially with regard to phosphorus (1.08 vs. 0.63 mg/L), although the final average concentrations were also lower in the SBR.

NMR analyses of the products from both reactors showed compounds frequently associated with anaerobic fermentation. Both reactors produced acetic acid as their primary product. The CBR had two products representing either isopropyl alcohol or an ether compound, although they could not be clearly distinguished from each other analytically (Table 2).

4.3 Effect of pH and loading on hydrogen production

CBR reactors were operated at various pH levels in order to determine the optimum. At least two reactors were run at each pH and the optimum was found to be about pH 5 (Fig. 3). This value is generally consistent with that determined for other hydrogen-producing systems [9, for review].

Various paper loadings were tested in order to approximate the level producing the most hydrogen per gram of paper. The optimum loading was around 4 g of paper per liter (Fig. 4).

4.4 Enzymatic source of hydrogen production: hydrogenase vs. nitrogenase

Since bacteria can produce hydrogen from reactions catalyzed by either hydrogenase or nitrogenase enzymes, an effort was made to estimate the relative contributions of the products of the two enzymes to the overall hydrogen yield. Reactors were run under similar conditions and purged with either nitrogen or argon. Argon-purged cultures, which were not provided nitrogen gas as a substrate for nitrogenase, averaged 55% of

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**Table 1 – Nitrogen and phosphorus removal efficiencies.**

<table>
<thead>
<tr>
<th></th>
<th>SBR</th>
<th>CBR</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Starting conc (mg/L)</td>
<td>Avg final conc (mg/L)</td>
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<tr>
<td>Nitrate</td>
<td>8.7</td>
<td>0.24</td>
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<td>Nitrite</td>
<td>0.035</td>
<td>0.01</td>
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<tr>
<td>Ammonia</td>
<td>0.53</td>
<td>0.18</td>
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<tr>
<td>Total nitrogen</td>
<td>9.27</td>
<td>0.44</td>
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<tr>
<td>Phosphorus</td>
<td>1.08</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Starting value (mg/L)</th>
<th>Average final value (mg/L)</th>
<th>Average % Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>8.0</td>
<td>0.19</td>
<td>98</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.19</td>
<td>0.01</td>
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<tr>
<td>Ammonia</td>
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<td>0.48</td>
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</tr>
<tr>
<td>Total nitrogen</td>
<td>8.32</td>
<td>0.69</td>
<td>92</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.63</td>
<td>0.28</td>
<td>56</td>
</tr>
</tbody>
</table>

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**Table 2 – NMR analyses of SBR and CBR products.**

<table>
<thead>
<tr>
<th></th>
<th>SBR Compound(s)</th>
<th>Mole %</th>
<th>CBR Compound(s)</th>
<th>Mole %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>75.7</td>
<td>Acetic acid</td>
<td>36.2</td>
<td></td>
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<tr>
<td>Propionic acid</td>
<td>14.9</td>
<td>Isopropyl alcohol or ether</td>
<td>24.1</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>7.2</td>
<td>Ethanol</td>
<td>22.6</td>
<td></td>
</tr>
<tr>
<td>Propanol, etc.</td>
<td>2.1</td>
<td>Butyric acid</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>Methanol (tentative)</td>
<td>0.09</td>
<td>Propionic acid</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isopropyl alcohol or ether</td>
<td>2.55</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 2 – Stability of hydrogen production: SBR vs. CBR.**

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**Fig. 3 – Hydrogen production as a function of pH.**

---

**Fig. 4 – Effect of paper loading on hydrogen production.**
the hydrogen output of the nitrogen-purged cultures (56.08 ± 2.8 ml H₂ per gram of paper vs. 101.98 ± 18.63 ml H₂ per gram of paper). The most straightforward explanation of these results could be that about half the hydrogen evolved from the cultures is produced as a byproduct of the fixation of nitrogen by nitrogenase, which is not occurring in the argon-purged cultures. This tentative conclusion was further supported by the results of a fermentation conducted under the same conditions in the presence of nitrogen gas but with 50 mM ammonium chloride. Nitrogenase activity is normally repressed in the presence of ammonia because of the metabolic cost in ATP to fix nitrogen. That fermentation produced 46.78 ml H₂ per gram of paper (46% of that measured in the absence of ammonium chloride). The fact that generally similar results were obtained with argon-purged cultures and in the presence of excess ammonium chloride suggest a strong role for nitrogenase (perhaps 50%) in the production of hydrogen in this system.

4.5. Cycle analysis — hydrogen, TOC, nitrogen and phosphorus

In order to better understand the overall process chemistry, a longer CBR cycle was run with periodic sampling to allow measurement of TOC, nitrogen, and phosphorus levels in addition to hydrogen production. Fig. 5 shows a comparison between hydrogen production, which occurred during approximately the first two days of the cycle, and TOC removal, which continued for about a week. The initial, lower TOC value was apparently due to the heterogeneity of the system prior to degradation of the paper by the added cellulase. Theoretically, the 4 g/L of paper added would have a calculated TOC value of 1778 mg/L. The reactor never reached this level due to of the nature of the SSF reactor in which the production of hydrogen and the hydrolysis of the cellulose occur simultaneously. One advantage of this type of system, in addition to its operation in a single tank, is that the microbial fermentation activity continually removes the product of the enzyme reaction, pulling the equilibrium of that reaction towards the monosaccharide products which are quickly utilized. The TOC profile in Fig. 5 shows that TOC continues to be removed several days after the apparent cessation of hydrogen production. It is possible of course, that hydrogen production may continue at a greatly reduced rate yielding a headspace concentration below the detection limit of the HY-OPTIMA™ 700 hydrogen monitor (0.5% hydrogen). Potentially, such lower levels of production could be determined by using a lower nitrogen gas flow rate.

Fig. 6 shows the nitrate, nitrite, ammonia, total nitrogen, and phosphorus profiles from the same run as shown in Fig. 5. Nitrogen content consisted mainly of nitrate and ammonia (starting concentrations 8.4 and 2.64 g/L, respectively). Nitrate and ammonia values were reduced to around 1 mg/L or less within the first day, while nitrite stayed relatively constant at around 0.2–0.3 mg/L. Phosphorus levels showed a similar magnitude of decline, going from 6.6 mg/L to around 1 mg/L (Fig. 7).

4.6. Hydrogen production rate

Using the data on headspace hydrogen concentrations over time, it is possible to determine hydrogen production rates for the system. The headspace volume was about 2 L for a reactor volume of 5 L, so the instantaneous changes in hydrogen production rates were necessarily somewhat averaged by the headspace volume and carrier gas flow rate (5 ml/min nitrogen). Hydrogen production rates over the course of the
reaction were calculated for the three highest producing CBR runs (138, 114 and 110 ml hydrogen per gram of paper, respectively) The maximum observed rate of hydrogen production was about 14 ml per L per hour, which was also observed with the culture yielding the greatest overall volume of hydrogen.

5. Discussion

With regard to reactor design, the CBR offers simpler operation with superior hydrogen yields over time, although somewhat less efficient nitrogen and phosphorus removal. The decline in hydrogen production in the SBR as compared to the CBR can probably, most simply, be explained by the accumulation of hydrogen-consuming organisms such as methanogens. These cultures were largely grown under nitrogen fixation conditions (with the exception of the initial consumption of the approximately 10 mg/L nitrogen found in the starting effluent) and nitrogen fixation is widespread in methanogenic organisms. Hydrogen-producing acidogenic bacteria are fast growers in comparison to methanogens [11] and the steady accumulation of biomass in the SBR may have gradually increased the predominance of methanogens in the culture. The CBR reactor on the other hand, produced hydrogen at a fairly steady level averaging a little over 100 ml H₂ per gram of paper.

The average hydrogen yield under optimal conditions (pH 5, 4 g/L loading, 0.45 ml/g Accellerase 1500, 38 °C) of 101.98 ± 18.63 ml per gram of paper equates to 4.52 ± 0.83 lbs of hydrogen per ton of paper.

One potentially significant variable not controlled for in the experiments described here is the concentration of hydrogen in the headspace of the reactor. Acidogenic bacteria, particularly those that produce acetic acid as a byproduct, are subject to feedback inhibition when hydrogen accumulates to even very low levels in the headspace [10]. This feedback might at least partially explain the relatively high variability in hydrogen yields observed under the most efficient conditions (4 g/L, pH 5), that typically provided for the overall highest concentrations of hydrogen in the headspace at any one time.

6. Conclusions

Dark fermentation of paper in WWTP effluent offers the potential to simultaneously produce hydrogen and remove nitrogen and phosphorus from wastewater. Used in the CBR configuration, the process is particularly simple in concept, requiring no sterilization or added nutrients. The optimized conditions developed with paper as a substrate may also convey to the use of a similar process with lignocellulosic biomass, although such biomass would likely require thermochemical pretreatment prior to enzymatic digestion and
fermentation. TOC removal data suggest the feasibility of linking this approach with a second stage, possibly using photosynthetic bacteria or a microbial fuel cell.

Acknowledgments

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