Biological hydrogen production from industrial wastewater with *Clostridium beijerinckii*

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Abstract

An investigation of biohydrogen production from glucose by *Clostridium beijerinckii* was conducted in a synthetic wastewater solution. A study examining the effect of initial pH (range 5.7 to 6.5) and COD loading (range 1 to 3 g/L) on the specific conversion and hydrogen production rate has shown interaction behaviour between the two independent variables. Highest conversion of 10.3 mL H₂/(g COD/L) was achieved at pH of 6.1 and COD of 3 g/L, whereas the highest production rate of 71 mL H₂/(h*L) was measured at pH 6.3 and substrate loading of 2.5 g COD/L. In general, there appears to be a strong trend of increasing hydrogen production rate with an increase in both substrate concentration and pH. Butyrate (14% to 63%), formate (10% to 45%) and ethanol (16% to 40%) were the main soluble products with other volatile fatty acids and alcohols present in smaller quantities. Absence of the key nutrients biotin, MgSO₄.7H₂O and FeSO₄.7H₂O caused a significant decrease in hydrogen yield when compared to the results obtained under standard synthetic wastewater conditions, though no significant difference was observed when concentrations of biotin, MgSO₄.7H₂O, K₂HPO₄, KH₂PO₄, were decreased partially. Preliminary experiments with wastewater effluent obtained from a yogurt manufacturer gave poor biohydrogen production.
Sommaire

Ce projet de recherche porte sur la production de biohydrogène par le *Clostridium beijerinckii* en utilisant comme substrat le glucose contenu dans une eau usée synthétique. Une étude de l’effet du pH initial (intervalle de 5.7 à 6.5) et de la charge organique (intervalle de DCO de 1 à 3 g/L) sur la conversion spécifique et le taux de production d’hydrogène a démontré des interactions entre ces deux variables indépendantes. La conversion la plus élevée, 10.3 mL H₂/(g COD/L), a été observée à un pH de 6.1 et une DCO de 3 g/L alors que le plus haut taux de production, 71 mL H₂/(h*L), a été obtenu à un pH de 6.3 et une DCO de 2.5 g/L. La tendance globale indique que le taux de production d’hydrogène augmente lorsque la charge organique et le pH augmentent. Le butyrate (14% à 63%), le formiate (10% à 45%) et l’éthanol (16% à 40%) formaient les principaux produits solubles. Des acides gras volatiles et des alcools ont également été observés en faibles quantités. L’absence de nutriments essentiels tels que biotine, le MgSO₄.7H₂O et le FeSO₄.7H₂O a causé une diminution significative de la production d’hydrogène comparativement aux résultats obtenus à l’aide des conditions de référence utilisées pour la solution d’eau usée synthétique. Aucun impact significatif n’a toutefois été observé lorsque la concentration de ces nutriments était diminuée que partiellement. Des essais préliminaires à partir d’eau usée provenant d’une usine de production de yogourt ont indiqué une faible production d’hydrogène à partir de ce substrat.
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1 Introduction

In the past few decades, the increasing energy consumption has put a strain on the supply of fossil fuels as well as on our planet and its ecosystems. The atmospheric concentration of greenhouse gases (GHGs) has been steadily rising and this increase has been directly linked to human activity. At a recent summit in Paris, France, the Intergovernmental Panel on Climate Change, comprised of over 600 scientists and climate change experts, concluded that the increases in the global carbon dioxide concentration are due predominantly to fossil fuel use [1] and called for an immediate action in the reduction of CO₂ emissions. Hydrogen has been identified as a possible alternative to fossil fuel energy and a worldwide investigation of hydrogen as a future energy carrier is now underway.

Current hydrogen generation methods can be grouped into two broad categories; conventional and alternative. Conventional hydrogen production methods mainly involve fossil fuel reforming, a process where, again, large amounts of CO₂ are generated. Alternative methods of hydrogen generation include electrolysis of water, biophotolysis and biological production from waste organic material. Of all the novel processes, biological hydrogen production has two main advantages over the conventional methods; it generates less GHGs and couples the metabolic activity of hydrogen-emitting micro-organisms with the simultaneous disposal of human-derived wastes rich in organics, such as domestic and food industry wastewaters.

Anaerobic (or dark) fermentation and photosynthetic degradation are the two most widely studied biohydrogen production techniques. Anaerobic fermentation is advantageous due to its rapid hydrogen evolution rate, while in the photosynthetic process complete conversion of organic matter is (theoretically) possible, allowing for total disposal of unwanted waste. Each process on its own, however, suffers from relatively low yields. It has been suggested that for biohydrogen production to be economically feasible, hydrogen yields on dissolved organic material must be approximately 60–80% [2]. Combining the
two methods, either directly or in a two step series-type configuration, may be a promising way to meet this conversion target.

A number of studies have examined the potential of using mixed communities of anaerobic bacteria obtained from anaerobic sludge digesters [3-5] and compost piles [6] in order to degrade both simple sugars such as glucose and sucrose and more complex substrates such as effluent from a sugary refinery [7]. There are a limited number of studies that deal with pure cultures of known species of hydrogen-producing bacterium [8, 9], however much can be learned about conditions which are favourable to high hydrogen yield and production rate by studying pure cultures of anaerobic bacteria.

Working with pure cultures eliminates the problem of bacterial community culture shift which is often a problem encountered when dealing with a population of mixed bacteria. Maintaining the uniformity of a culture over time ensures that the differences observed throughout the experiment are a true representation of the variables studied and are not related to the bacterial culture. Furthermore, metabolic shifts are more easily detected in pure culture systems due to the reduced complexity of the matrix in question. Finally, the logistics involved in genetic manipulation/modification in creating a more “active” hydrogen producing bacteria are much less intensive when dealing with a pure bacterium, as the isolation step is not necessary.

The goal of this project was to examine best conditions (pH, nutrient content and substrate loading) for hydrogen production for a pure culture of anaerobic bacteria in synthetic wastewater on a batch scale, in hopes of developing a continuous fermentative hydrogen production process and eventually, a two-stage production set-up with the fermentative and photosynthetic methods in a series arrangement. The motivation is that, if we can achieve a sufficiently high production rate and yield of hydrogen from industrial wastewater, we can develop an on-site system which can simultaneously treat the effluent prior to disposal and recover part of the energy stored in the dissolved organic waste.
1.1 Objectives

The purpose of the project was to investigate the potential of hydrogen production from wastewater (synthetic and industrial) using a pure culture of \( C. \) \textit{beijerinckii} (anaerobic) bacteria. The specific objectives of this project were as follows:

1) To develop laboratory techniques necessary for the cultivation of anaerobic organisms and biological hydrogen production experiments;

2) To investigate the effect of initial pH and substrate concentration (glucose) on the specific \( \text{H}_2 \) production potential and maximum production rate on the batch scale;

3) To identify and quantify the main soluble metabolites of the fermentation process;

4) To study the effect of varying concentration of key nutrients, biotin, \( \text{MgSO}_4.\text{H}_2\text{O} \), \( \text{K}_2\text{HPO}_4 \), \( \text{KH}_2\text{PO}_4 \) and \( \text{FeSO}_4.\text{H}_2\text{O} \), on the hydrogen production;

5) To conduct a preliminary assessment of the suitability of raw industrial wastewater as a potential feedstock for biohydrogen production.

1.2 Hypotheses

Biological hydrogen production utilizing a mixed consortium of anaerobic bacteria using various substrates has been successfully conducted and widely documented in many laboratory scale studies, both in batch and semi-continuous/continuous arrangements. Parameters such as initial pH, type of substrate, substrate loading and hydraulic retention time have been studied in order to establish most promising operating conditions. Based on these studies, the specific hypotheses of the project were as follows:

1) The initial pH and substrate concentration will have a significant effect on both the specific yield and volumetric production rate of hydrogen, with more acidic pH and higher glucose concentration being favourable to higher hydrogen yield and greater maximum rate of production;
2) Organic acids will be the main soluble metabolites of the process (mainly acetic and butyric acids);

3) Absence of key nutrients will have a negative effect on both hydrogen yield and rate of production, while increasing the concentration of FeSO₄·7H₂O will give rise to greater hydrogen gas evolution;

4) Assuming the proper level of COD is present, industrial wastewater will prove to be a suitable feedstock for hydrogen production, though nutrient addition will most likely be necessary.

1.3 Significance

The rising costs in conventional energy supplies and the established link between climate change and the burning of fossil fuels have revitalized the search for alternative fuels and modes of energy production. Options where disposal of unwanted wastes can be coupled with a novel energy production technique are particularly desirable. Policymakers are beginning to recognize the importance of addressing climate change issues; as part of their ecoEnergy Technology Initiative, the Canadian government recently announced a commitment of 230 million dollars, over four years, to research and development projects which concentrate on reduction of GHG emissions and advancement of clean and renewable energy technologies.

The results obtained from this work could provide a stepping stone for the development, within the Canadian private sector, of full-scale biohydrogen production systems that are less energy intensive and more environmentally friendly than conventional methods. Understanding the role of key parameters (pH and substrate concentration) in biological hydrogen production on a batch scale, can allow for a more effective scale-up and implementation of larger continuous processes. Assessing the potential of industrial wastewater as feedstock for hydrogen production can lead to the development of on-site hydrogen production systems where wastewater treatment and fuel/energy generation are achieved simultaneously.
2 Literature Review

2.1 Rationale for studying renewable energy sources

Since the industrial revolution humans have been heavily dependent on fossil fuels for their energy source and, with the growing economy and globalization, that dependence is only expected to intensify. In 2003 the Energy Information Administration (EIA) estimated the total world energy consumption at 420.7 quadrillion BTU, a number which is projected to increase to almost 510 quadrillion BTU by the year 2010 [10]. This continuing demand has already put an enormous strain on the available fossil fuel reserves.

In the 2005 World Energy Report, the German Federal Institute for Geosciences and Natural Resources (BGR) estimated that more than 60% of the total potential petroleum has been extracted in North America and almost 50% in Western Europe [11]. Similarly, the total global natural gas (NG) reserves were reported at 6112 trillion cubic feet as of January 1, 2006, and the 2003 global annual NG consumption was 95 trillion cubic feet [10]; without accounting for the growth in demand, this would suggest that this resource will run out in approximately 60 years.

Similar trends are occurring in the petroleum sector; Figure 2.1 shows the global petroleum consumption over approximately a 30 year span. It is clear from the graph that the worldwide demand for oil is growing; over the last 15 years the petroleum industry has experienced roughly a 30% spike in oil use. Considering that both China and India are experiencing rapid industrialization it is not difficult to envision a potential oil shortage crisis within in the next few decades. In Canada, fossil fuel use has increased by 20% since the early 1990s and the national demand for hydrocarbon fuels is expected to continue to rise [12]. Despite the claim by some that evolving technology will allow us to tap into currently unavailable energy sources, it is becoming clearer each day that our present reliance on finite energy resources cannot be sustainable in the long term.
Figure 2.1: Global petroleum consumption from 1975 to 2007 [13].

The increasing energy consumption has not only put a strain on our raw resources, but also on our planet and its ecosystems. Incomplete combustion processes, which result from the burning of fossil fuels, produce a great amount of gases such as carbon dioxide (CO$_2$) and nitrogen oxides (NO$_x$). Carbon dioxide emissions are of particular concern, since CO$_2$ has been identified as a greenhouse gas (GHG). The atmospheric concentration of GHGs has been steadily rising and in 2005 the concentration of CO$_2$ rose to a record high of 378.9 ppm [14]. This increase has been directly linked to human activity; over 25,000 metric tons of CO$_2$ were emitted in 2003 [10], a value that is projected to grow over the years. In 2005, Canada ranked fourth worst among 30 OECD countries with regards to CO$_2$ emissions at 17.00 tonnes of CO$_2$/capita [15]. High concentrations of GHGs have resulted in a rise of the average surface temperatures as well as adverse effects on weather patterns, human and animal life.

Climate change, along with the rapid depletion of oil and gas reserves, has prompted many to search for environmentally friendly energy alternatives, ideally ones from renewable sources. Hydrogen has been identified as a clean energy carrier and one potential alternative to fossil fuel energy.
2.2 **Hydrogen production: conventional and novel approaches**

Hydrogen is the most abundant element on Earth; however it is not easily accessible. Molecular hydrogen is present in fossil fuels, water, as well as most organic compounds and as such must first be extracted from the raw material and converted to its diatomic form. The current hydrogen production methods can be grouped into three main categories: fossil fuel processing which includes natural gas, coal, and biomass as the process input; electrolysis of water using conventional, renewable, or nuclear energy for the electricity source; and modern approaches which include photo-synthetic and photo-biological splitting of water [16, 17]. Figure 2.2 depicts the various hydrogen production methods in greater detail.

![Hydrogen production methods diagram](image)

**Figure 2.2: Hydrogen production methods:** Fossil-fuel processing, biological and other current and novel approaches.

2.2.1 **Fossil Fuel Reforming**

In 2003, over 95% of the global hydrogen was produced through natural gas, oil, or coal reforming [17]. In general all these processes involve the need for high temperatures and/or pressures (as in the case of coal gasification) as well as the production of other gases, mainly carbon dioxide. Partial oxidation of natural gas as well as coal gasification methods generate less CO₂ than conventional
internal combustion engines [18], however from an environmental perspective all these processes fail to resolve the issue of reducing the total CO₂ emissions. Furthermore, due to the energy intensive nature of hydrogen production via reforming, the cost of one unit of energy delivered from hydrogen is more costly than the same unit of energy obtained from using the raw hydrocarbon in the first place.

In 2002 the United States Department of Energy put forth a National Hydrogen Energy Roadmap report [19] outlining the prospects and challenges associated with shifting towards a hydrogen based energy economy. The report stressed that for this energy shift to be economically viable, hydrogen production via methods alternative to those dependent on fossil fuels, need to be more mature and urged that more efforts, both in the private and public sectors, be devoted to research and development of novel and sustainable hydrogen production techniques.

2.2.2 Electrolysis

Electrolysis of water involves passing an electric current through water and thus splitting it into oxygen and hydrogen gas. After fossil fuel reforming, electrolysis is the most utilized method of hydrogen production [17, 20]. Typically large amounts of energy are required for the process, which makes electrolysis the most costly method of hydrogen production. The positive aspect of this approach is that it does not produce any GHGs, however its overall “environmental-friendliness” is highly dependent on the source used for electricity generation. Hydrogen produced in this manner has a potential of being completely emission free if electricity is generated using a renewable source such as wind or solar [17, 18, 20]. Currently, however, electrolysis cannot compete with reforming processes in large volume production.

2.2.3 Biomass gasification

Biomass gasification is similar to conventional fossil fuel reforming but has the added benefit of using waste sludge material as the process input. The availability of biomass in Canada is substantial; an average annual commercial
harvest of forest and agricultural biomass is approximately $1.43 \times 10^8$ t carbon [21]. When translated into energy, this figure has the potential energy content of approximately $2.25 \times 10^9$ GJ, or roughly 22% of Canada’s annual fossil fuel demand. Due to the costs as well as the impracticality associated with the transportation of the biomass to a centralized location, however, it is unlikely that this method will ever be employed in anything but small-scale applications (ex. on a farm where biomass is readily available). More importantly, expensive equipment as well as other economically competitive options for biomass use make the gasification process, at best, marginally cost-effective [22]. The use of biomass as feedstock for a biologically based hydrogen production process is more attractive both in the potential for large applications and economical viability.

2.3 Biological hydrogen production

In nature, only bacteria or microalgae have the ability to produce hydrogen [23]. Biological hydrogen production has an advantage over the conventional methods in that it utilizes these plentiful and naturally occurring micro-organisms as catalysts and eliminates the need for high temperature, energy intensive reactor operation. All bacteria and algae require energy and a carbon source to carry out their cellular functions. In the cases where the carbon source must be organic, this activity can be coupled with the treatment and disposal of human-derived wastes rich in organics, such as various biomass residues and domestic or food industry wastewaters.

Several approaches have been used to try and take advantage of the bacterial hydrogen producing metabolism. Currently, the main processes for biohydrogen production include: 1) biophotolysis of water using algae/cyanobacteria, 2) photodecomposition of organic compounds using photosynthetic bacteria, 3) fermentative hydrogen evolution using anaerobic bacteria and 4) hybrid systems combining the fermentative and photosynthetic approaches either directly or in a series-type configuration. A novel technology which focuses on combining the biological hydrogen production and electricity generation inside a microbial-fuel
cell (MFC) has been recently proposed and will be discussed, alongside the other aforementioned methods, in greater detail in the following sections.

2.3.1 Biophotolysis – green algae/cyanobacteria

Out of all the biohydrogen production processes, biophotolysis is theoretically the most appealing. In biophotolysis, algae or cyanobacteria (green-blue algae) decompose water to hydrogen and oxygen with the aid of sunlight according to the following reaction:

\[ \text{H}_2\text{O} \rightarrow 2\text{H}^+ + \frac{1}{2}\text{O}_2 \]

In the case of the unicellular algae, the hydrogen is generated via the hydrogenase enzyme as depicted in Figure 2.3. In the case of the cyanobacteria the water splitting process involves two enzymes; hydrogenase and nitrogenase both of which catalyze the hydrogen generation process. Since only water, sunlight, and atmospheric CO\(_2\) (carbon source) are involved in the process, biophotolysis is very attractive from an environmental point of view.

\[ \text{H}_2\text{O} \rightarrow \text{PSII}^a \rightarrow \text{PSI}^b \rightarrow \text{Ferredoxin} \rightarrow \text{Hydrogenase} \rightarrow \text{H}_2 \]

\[ \downarrow \]

\[ \text{O}_2 \]

\(a\) – photosystem (PS) II, responsible for the water splitting and O\(_2\) evolution

\(b\) – photosystem I, responsible for generation of the reductant for CO\(_2\) reduction

**Figure 2.3: Hydrogen production pathway of unicellular algae [24].**

There are, however, a number of limitations to making this method economically attractive. The solar conversion efficiency of this process is approximately 10%, resulting in the need for large bioreactor surface area, a large reactor foot-print and thus high capital costs [25, 26]. Furthermore, the overall rate of hydrogen evolution is slow as the algal hydrogenase is inhibited by oxygen which is generated during the process [26-28]. Similarly, in the case of the
cyanobacteria, the photosynthetically generated O₂ irreversibly inactivates the H₂ producing system and activates the oxygen-dependent, hydrogen-uptake activity [24].

Efforts have been made to genetically modify the microorganisms to obtain a system less sensitive to oxygen, but to date there are no known significant successes in this field. Though fundamentally attractive, biophotolysis has been deemed by some [22] as economically unfeasible due to its oxygen sensitivity, poor light conversion efficiencies and slow hydrogen production rate.

2.3.2 Biocatalyzed electrolysis

Biocatalyzed electrolysis is a novel technology that utilizes electrochemically active micro-organisms which, with a small voltage input, convert dissolved organic matter into hydrogen inside an electrochemical cell/microbial fuel cell via coupled anode-cathode reactions [29]. The advantage of this system is that the energy stored in waste streams can be directly recovered as electricity. In their recent work, using acetate as a substrate, Rozendal et al [23] produced 0.02 m³ H₂/m³ reactor liquid volume/day in a biocatalyzed electrolysis set up, yielding an overall conversion efficiency on substrate of ~55%. The authors postulated that improvements of the experimental set-up as well as process optimization would allow for future volumetric H₂ production rates >10 m³ H₂/m³. More research is needed in addressing key limitations such as biocatalyst activity, electron transfer between the bacteria and anode and internal resistance of the MFCs [29]. Nonetheless, biocatalyzed electrolysis appears to be a promising future approach to hydrogen generation from wastewater, especially for effluents with high organic content.

2.3.3 Photosynthetic production

Photosynthetic bacteria have long been studied for their capacity to produce significant amounts of hydrogen due to their high substrate conversion efficiencies and ability to degrade a wide range of substrates. Photoheterotrophes utilize dissolved organic compounds, as well as sunlight, and produce hydrogen under anaerobic/anoxic conditions. Theoretically, these
bacteria are capable of complete conversion of various organic substances to produce hydrogen and carbon dioxide, as shown (with glucose) in the following reaction:

\[
\text{Glucose} + 6\text{H}_2\text{O} \rightarrow 12\text{H}_2 + 12\text{CO}_2
\]

As a result, much work has been devoted to the development of systems which utilize photo-heterotrophic bacteria for simultaneous hydrogen generation and waste disposal.

Photosynthetic bacteria, such as \textit{Rhodobacter Sphaeroides}, generate hydrogen mainly through the action of the nitrogenase enzyme. This activity is inhibited by the presence of oxygen, excess amounts of ammonia or high nitrogen/carbon ratios [30-32]. In fact, high nitrogen concentrations have been linked to a metabolic shift where organic substrate is utilized mainly for biomass generation instead of hydrogen production [33]. High biomass concentration is not desirable due to the reduction of light diffusion into the bioreactor.

As with all biological systems, careful consideration must be given to operating conditions in order to maximize the desired parameters, in this case hydrogen yield and rate of production. Depending on the temperature, pH, the bacterial strain, irradiation and substrate used, \textit{R. Sphaeroides} has exhibited various results. The optimum temperature and pH are in the range of 30-35°C and 7.0-8.0, respectively and irradiation of approximately 200 W/m² [9, 30, 34-36]. Though hydrogen production has been achieved with \textit{R. Sphaeroides} growing on various substrates, the bacteria seem to prefer organic acids such as acetic, butyric [9, 36, 37], malic [30, 35, 36] and lactic acid [34-36]. Utilization of simple sugars such as glucose and sucrose for hydrogen production is possible, but usually results in lower substrate conversion and rate of H₂-evolution [35] and is secondary if an organic acid co-substrate is present [9]. Some success in using industrial wastewater as substrate has been shown [7, 38], but due to either the toxic nature of the effluent or colour/opaqueness, pre-treatment is needed prior to photosynthetic biohydrogen gas production.
Despite the successes of hydrogen generation via photosynthetic degradation of organic compounds, much work is still needed to create a large-scale, economically attractive process. Though the conversion of substrate is generally high, the production rate of $H_2$ is slow and the yield of hydrogen is far from the theoretical maximum. As with other light-based $H_2$ production processes, light diffusion and intensity play a key role in maximizing hydrogen yield. Increasing light intensity (to a certain threshold) increases the hydrogen yield and production rate, but has a negative effect on the light conversion efficiency. Expensive equipment and the need for large reactor surface area also remain a drawback. Though cyclic light process operation (i.e. light-dark cycles) has been shown to increase the amount of hydrogen evolved when compared to continuous illumination [32], many questions remain about whether overall light conversion efficiencies are high enough to warrant large-scale systems. Photosynthetic hydrogen production might have to be coupled with another process in order to make it an economically viable option of biogas production.

2.4 Anaerobic (dark) fermentation

Anaerobic fermentation is similar to the photosynthetic process in that it makes use of micro-organisms to produce hydrogen from organic matter. Anaerobic systems have an advantage over their photosynthetic counterparts in that they are simpler and less expensive and produce hydrogen at a much faster rate. The drawback, however, is that anaerobes are unable to utilize light energy and thus lack the ability to overcome the inherit thermodynamic energy barrier to fully decompose a substrate [25]. As with many other hydrogen producing organisms, the hydrogenase enzyme is responsible for catalyzing the hydrogen generating reaction in anaerobic bacteria. Anaerobes break down substrates (mainly carbohydrates) to acetic and butyric acids (i.e. acetate and butyrate), with theoretical yields of four and two moles of hydrogen per each mole of glucose as shown below:
Environmental conditions affect these metabolic pathways and the presence of acetate and butyrate is typically accompanied by other volatile fatty acids (VFAs) and alcohols. Figure 2.4 shows a few other common metabolic routes that can occur during fermentative hydrogen production, which do not yield any hydrogen (ex. ethanol pathway). As can be seen, hydrogen is derived from pyruvate, which is formed during the breakdown of glucose. The pyruvate is then catalyzed by pyruvate ferredoxin (flavodoxin) oxidoreductase (PFOR), under anoxic conditions, to produce acetylCoA and reduced ferredoxin (Fd(red)); ATP can be obtained from the former, while hydrogen is derived from Fd(red) via the hydrogenase [26, 39]. The byproducts of the fermentation include, ethanol, propanoic and lactic acids; these substances represent hydrogen that has not been released as gas. In the case where pyruvate breakdown is catalyzed by pyruvate formate lyase (PFL), production of acetylCoA is simultaneous with formate generation (not shown). In general, strict anaerobic bacteria produce hydrogen through the oxidation of Fd(red) [26].

\[
\begin{align*}
\text{C}_6\text{H}_{12}\text{O}_6(s) + 2\text{H}_2\text{O} & \rightarrow 2\text{CH}_3\text{COOH}_{(aq)} + 2\text{CO}_2(g) + 4\text{H}_2(g) & \text{(A)} \\
\text{C}_6\text{H}_{12}\text{O}_6(s) & \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}_{(aq)} + 2\text{CO}_2(g) + 2\text{H}_2(g) & \text{(B)}
\end{align*}
\]

![Figure 2.4: Metabolic pathways in fermentative hydrogen evolution [39].](image-url)
Understanding the relationship between bacterial metabolisms and operating conditions is critical to increasing the desired product output, in this case hydrogen. In general, anaerobic systems suffer from low hydrogen yields. The reason for this is that anaerobes are optimized, evolutionarily, for maximizing biomass and not hydrogen [26]. Also, many anaerobic organisms contain an uptake hydrogenase which utilizes hydrogen gas and thus reduces the actual yields of hydrogen [40]. In order to maximize hydrogen yield, substrate metabolism should be steered towards VFAs and away from alcohol (solventogenesis) or reduced acid production (ex. lactate). Typically, anaerobic species (ex. from the genus Clostridium) generate gas in the exponential growth phase and the metabolism shifts from H$_2$/acid production to solventogenesis when the culture population reaches stationary growth phase [40, 41]. Poor hydrogen yields have also been linked to high hydrogen partial pressure, high substrate concentration, low iron concentration and/or low pH [41-43].

Current maximum hydrogen yields obtained from anaerobic bacteria do not make the fermentative process an attractive one from an economical point of view when compared to the conventional reforming techniques. Ongoing research is attempting to address this issue and identify a set of parameter under which both yield and production rate can be maximized. The following sections will review key fermentative parameters for maximizing hydrogen yield and production rate, as well as the feasibility of using various renewable feedstocks, particularly wastewater, for biohydrogen production. The incorporation of a hybrid/second stage process of is also discussed.

2.4.1 Bacteria studied

Organisms belonging to the genus Clostridium, such as C. butyricum, C. beijerinckii, C. acetobutyricum and C. bifermentans are strict, spore forming anaerobic bacteria. The majority of studies involving anaerobic hydrogen production have involved the use of Clostridium bacteria; high yields have been obtained using inoculum from pure cultures, mixed anaerobic communities where Clostridia were shown to be the dominant organisms, as well as individual strains isolated from waste material [5, 28, 36]. A recent study by Jeong et al [44]
comparing four different kinds of anaerobic bacteria (*R. sphaeroides*, *B. megaterium*, *C. beijerinckii* and mixed anaerobic bacteria) found the pure *C. beijerinckii* as the best producer from glucose in terms of specific hydrogen production potential and hydrogen production rate. The downside of using a pure strain is that sterile and anaerobic conditions should be maintained throughout the experiment, which may prove difficult on a larger industrial scale [41]. Industrial wastewater, particularly from a food processing facility, could potentially prove to be an economically attractive feedstock due to high sugar content, low bacterial concentration and relatively clean effluents. The drawback of using a mixed culture inoculum, on the other hand, is that a culture shift can occur after a certain period of continuous operation causing a drop in hydrogen yield and rate of production.

*Enterobacter aerogenes* is a facultative bacterium capable of anaerobic hydrogen production. In a recent study, Ogino *et al* [28] used a pure culture of *E. aerogenes* to produce hydrogen from a wide range of substrates including glucose, sucrose and dextrin. The yield of hydrogen, however, is generally higher from the *Clostridium* species than from *E. aerogenes* [41]. As with all strict anaerobic bacteria, small concentrations of oxygen in the liquid can inhibit *Clostridia* activity. For this reason, the inclusion of an expensive reducing agent into the liquid is necessary when working with pure strains; as a result, large scale hydrogen production using a pure culture may prove to be not economically feasible. Yokoi *et al* [45] used a mixed culture of pure *C. butyricum* and *E. aerogenes* without the addition of a reducing agent and achieved a nearly 50% increase in hydrogen production when compared to *C. butyricum* with reducing agent alone. Similar results were reported elsewhere [28]. Thus, the use of *E. aerogenes* in conjunction with *Clostridium* could simultaneously resolve the need for addition of expensive reducing agents and increase hydrogen production.

2.4.2 Parameters affecting hydrogen production

Anaerobic fermentations have been studied extensively over the past 20 years though mainly for the production of butanol, acetone and ethanol. As such, utilizing anaerobes is for the production of biohydrogen is a relatively new
concept and much work is still needed in optimizing the operating conditions to attain maximal hydrogen yields and production rates. Some of these include pH, type and concentration of substrate and nutrient availability.

2.4.2.1 pH

Out of all the parameters affecting specific hydrogen production potential (conversion efficiency) and rate of production, initial pH may prove to be of most importance. The pH not only affects the hydrogenase enzyme but can play a part in cell morphology and therefore biomass flocculation and cell adhesion [46]. Experiments have demonstrated that the optimal pH for cell growth does not appear to be the same as that for obtaining high hydrogen potential [41]. For the degradation of simple substrates, the optimum pH for the *Clostridium* genus has been reported in the range of 4.5 – 7.0 [5, 6, 41, 46, 47], though high yields have been reported at a pH value as large as 9.0 [46].

Setting the initial pH dictates a delicate balance between obtaining optimum conversion efficiency, and acquiring the fastest rate of hydrogen production. A pH value outside of the acceptable range can inhibit hydrogen production by altering bacteria’s metabolism or cause a microbial population shift (mixed culture inoculum) bringing about a termination in hydrogen production and as such, reliable pH control is crucial. Lay *et al* [48] has suggested that a pH of 5.6 is the optimum and dividing line between hydrogen/acid production and solventogenesis for an unidentified anaerobic inoculum.

In general, it has been shown in both batch and continuous experiments that the initial pH has a significant effect on both the yield and rate of hydrogen production, however, the trends are not consistent from one author to the other. Zhao and Yu [46] saw an increase in both the H₂ yield and production rate with an increasing pH in their work with a continuous reactor using sewage sludge and sucrose as inoculum and substrate, respectively. Both parameters reached maximum values at a pH of 7.0 (1.61 mol H₂/mol glucose and 144 mL H₂/hr) after which the trend reversed. Similar trends were observed in a study by Van Ginkel *et al* [6] in their work with various naturally-occurring inocula; the authors found that both production rate and hydrogen yield increase with increasing pH.
up to a certain maximum when using potato soil as inocula. Conversely, when compost inoculum was used, the trends completely reversed; lower pH gave rise to both higher hydrogen yield and rate of production. Different trends still were observed in the works by Khanal et al [47] and Chen et al [5]; in both studies the authors witnessed a decrease in H$_2$ yield with increasing pH. And while Khanal et al [47] saw a similar trend for production rate as function of pH as Ginkel et al [6] with potato soil, Chen et al observed the reverse effect [5]. All these results strongly imply that in scale-up processes, where high production rates are ideal, the optimum pH will need to be carefully assessed for the specific application in question.

### 2.4.2.2 Types of substrates and organic loading

Many studies have examined the hydrogen production potential of different carbon sources from simple sugars such as glucose to more complex substrates such as food processing wastewater. A brief summary of yields and rates of biohydrogen production (batch and continuous) on various simple substrates is shown in Table 2.1 and Table 2.2 (Note: reported rates and yields may not have occurred under same operating conditions). Saccharolytic bacteria from the genus Clostridia, will preferentially degrade carbohydrates over other complex molecules like lipids or amino acids. Glucose and sucrose are the most common pure substrate used in both batch and continuous processes, due to their relatively simple structures, ease of biodegradability and presence in most industrial effluents [40]. In general, the maximum yield on substrate by mixed/pure inoculum for both types of systems is in the range of 45 - 60% [49] under atmospheric/near atmospheric operating pressures regardless. This is based on the assumption that 4 moles of hydrogen are produced for each mole of glucose.

A small number of authors have studied the effect of substrate concentration on the biohydrogen production rate and yield. Chen et al [5] varied the sucrose concentration from 5 to 20 g COD/l to obtain an increasing hydrogen yield and production rate (up to 160 ml H$_2$/h l), however both values decreased when the substrate concentration reached 30 g COD/l. Similar results were
observed elsewhere [6, 50] though the inhibition occurred at a different substrate concentration. These results suggest that elevated substrate loadings have a toxic effect on the bacteria.

Table 2.1: Rates and yields of biohydrogen production from pure carbohydrates by anaerobic bacteria in a batch set-up.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Production rate</th>
<th>H₂ yield</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture (soil)</td>
<td>Glucose (4 g COD/L)</td>
<td>0.92 mol/mol S*</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>C. butyricum</td>
<td>Glucose (2% (w/v))</td>
<td>41 mL/(L*h)</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td>C. butyricum</td>
<td>Glucose (28 mM)</td>
<td>4.92 mL/day/mL</td>
<td>0.5 mL/mL</td>
<td>[9]</td>
</tr>
<tr>
<td>C. beijerinckii</td>
<td>Glucose (20 g/L)</td>
<td>90.35 mL/(L*h)</td>
<td>14.5 mL/g S</td>
<td>[44]</td>
</tr>
<tr>
<td>C. butyricum</td>
<td>Sucrose (20 g COD/L)</td>
<td>137 mL/(L*h)</td>
<td>2.78 mol/mol S</td>
<td>[5]</td>
</tr>
<tr>
<td>Mixed culture (compost pile)</td>
<td>Sucrose (7.5 g COD/L)</td>
<td>74.7 mL/(L*h)</td>
<td>38.9 mL/(g COD/L)</td>
<td>[6]</td>
</tr>
<tr>
<td>Mixed culture (compost pile)</td>
<td>Sucrose (10 g/L)</td>
<td>9.5 mL/hr/g VSS</td>
<td>214 mL/g COD</td>
<td>[47]</td>
</tr>
<tr>
<td>Mixed culture (soil)</td>
<td>Sucrose (4 g COD/L)</td>
<td>1.8 mol/mol S</td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>Mixed culture (compost pile)</td>
<td>Starch (10 g/L)</td>
<td>4.5 mL/hr/ g VSS</td>
<td>125 mL/g COD</td>
<td>[47]</td>
</tr>
<tr>
<td>Mixed culture (soil)</td>
<td>Potato starch (4 g COD/L)</td>
<td></td>
<td>0.59 mol/mol S</td>
<td>[51]</td>
</tr>
</tbody>
</table>

*S: substrate

Table 2.2: Rates and yields of biohydrogen production from pure carbohydrates by anaerobic bacteria in a continuous set-up.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Production rate</th>
<th>H₂ yield</th>
<th>Reactor</th>
<th>HRT (h)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture</td>
<td>Glucose (7 g/L)</td>
<td>191 mL/(g VSS*h)</td>
<td>2.1 mol/mol S</td>
<td>CSTR</td>
<td>6</td>
<td>[49]</td>
</tr>
<tr>
<td>Mixed culture (sewage sludge)</td>
<td>Sucrose (20 g COD/L)</td>
<td>53.5 mmol/(g VSS*day)</td>
<td>1.5 mmol/mol S</td>
<td>UASB</td>
<td>8</td>
<td>[52]</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Sucrose (10 g COD/L)</td>
<td>105 mL/(L*h)</td>
<td>1.19 mol/mol S</td>
<td>UASB</td>
<td>17</td>
<td>[53]</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Sucrose (10 g COD/L)</td>
<td>145 mL/(L*h)</td>
<td>1.61 mol/mol glucose</td>
<td>UASB</td>
<td>13</td>
<td>[46]</td>
</tr>
<tr>
<td>Mixed culture (digester sludge)</td>
<td>Sucrose (10 g/L)</td>
<td>1.7 mol/mol hexose</td>
<td>CSTR</td>
<td>12</td>
<td>[50]</td>
<td></td>
</tr>
</tbody>
</table>

*S: substrate

Though biohydrogen production from simple sugars is well researched, only a handful of studies deal with using industrial/domestic wastewater as a potential feedstock. These are summarized in Table 2.3. (Note: reported rates and yields may not have occurred under same operating conditions). In a recent study, Venkata et al [54] tested the ability of a composite chemical wastewater (CW), in
conjunction with co-substrates, to produce biohydrogen with a mixed culture; the CW was an aggregate of drugs/pharmaceuticals, pesticides and numerous chemical processing units and synthetic wastewater (SW) containing glucose (2 g/L) and nutrients, domestic sewage wastewater (DSW) and glucose served as co-substrates. The authors found that a 40%/60% mixture of CW/DSW gave the highest yield and highest relative H₂ production rate, followed by a 40%/60% mixture of CW/SW+1 g/L glucose. Interestingly, synthetic wastewater alone showed poor hydrogen evolution as did increasing the glucose co-substrate concentration. It has also been shown that addition of trace nutrients does not consistently increase hydrogen gas production [55, 56].

Table 2.3: Rates and yields of biohydrogen production from industrial effluents in continuous and batch-set ups.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Carbon source</th>
<th>Production rate</th>
<th>H₂ yield</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predominantly <em>C. pasterianum</em></td>
<td>Citric acid WW (15 – 10 g COD/L)</td>
<td>0.72 m³/(m³*day)</td>
<td>0.84 mol/mol hexose removed</td>
<td>[57]</td>
</tr>
<tr>
<td>Mixed culture (sludge)</td>
<td>40% Chemical WW (6.24 g COD/L) + 60% Domestic WW (0.43 g COD/L)</td>
<td>0.0835 mmol/(g COD*h)</td>
<td>1.25 mmol/g COD</td>
<td>[54]</td>
</tr>
<tr>
<td>Mixed culture (sludge)</td>
<td>40% Chemical WW (4.5 g COD/L) + 60% Synthetic WW + 1 g glucose</td>
<td>0.0298 mmol/(g COD*h)</td>
<td>1.15 mmol/g COD</td>
<td></td>
</tr>
<tr>
<td>Mixed culture (soil)</td>
<td>Potato processing WW (21 g COD/L)</td>
<td>0.21 L/(L*h)</td>
<td>0.14 L/g COD</td>
<td>[55]</td>
</tr>
<tr>
<td>Mixed culture (soil)</td>
<td>Apple processing WW (9 g COD/L)</td>
<td>0.09 L/(L*h)</td>
<td>0.1 L/g COD</td>
<td>[55]</td>
</tr>
<tr>
<td>Mixed culture (sludge)</td>
<td>Dairy WW (10.4 g COD/L)</td>
<td>7.37 mmol/(m³*min)</td>
<td>0.034 mmol/min</td>
<td>[58]</td>
</tr>
<tr>
<td>Mixed culture (settling tank)</td>
<td>Rice winery WW (29.5-35.4 g COD/L)</td>
<td>9.33 L/(g VSS* day)</td>
<td>2.14 mol/mol hexose</td>
<td>[43]</td>
</tr>
<tr>
<td>Mixed culture (sludge)</td>
<td>Cereal processing WW (8.9 g COD/L)</td>
<td>5.7 mL/(L*h)</td>
<td>0.71 mol/mol glucose</td>
<td>[56]</td>
</tr>
</tbody>
</table>

In a different study, Venkata et al [58] showed the suitability of using dairy wastewater for biohydrogen production. Not only were the authors able to achieve significant hydrogen production in a continuous set-up, but also accomplish high COD removal efficiencies (>60% in some cases) proving that the dairy wastewater participated as the main carbon source for the bacteria. Similar
results were obtained elsewhere [56, 57]. Generally, such removal efficiencies are acceptable for a first stage of wastewater treatment.

2.4.2.3 Nutrient concentrations

Inorganic nutrients such as N, P, S and Fe are essential to maximizing hydrogen production. The latter two are critical components of the hydrogenase and as such must be present in sufficient concentration to ensure proper function of the enzyme. Studies have shown, that iron limitations lead to significant reduction and sometimes cessation of hydrogen production in anaerobic bacteria, as low iron concentrations favoured ethanol and butanol production [41, 42, 59].

Nearly all studies reported in the literature include the use of a complex mineral salt solution in conjunction with the primary carbon source. Trace metals and vitamins such as Ca, Mo, Zn, Cu, Cu, Mn and vitamin B are often included into the recipe at various concentrations and despite high costs related to some of these species, little work has been dedicated to identifying their lowest necessary concentrations [41]. Minimum amounts of trace nutrients needed for efficient and economical biohydrogen production under varying operating conditions (ex. pH, substrate concentration, etc.) are yet to be determined.

2.4.3 Hybrid systems: fermentative and photosynthetic

It has been argued that in order for hydrogen production by dark fermentation to be economically feasible and sustainable, a two-step/hybrid biological hydrogen production process would be necessary [2]. By combining the anaerobic and photosynthetic steps, higher overall substrate conversion efficiency is possible as the photosynthetic microbes can degrade the soluble metabolites from the fermentative step using sunlight to overcome the energy barrier as shown in Figure 2.5. VFAs are the main soluble breakdown products from the first step and these are preferred substrates of photo-heterotrophic bacteria [9, 36, 37].
Theoretically, 12 moles of hydrogen can be produced from 1 mole of glucose in the two step process. Eroglu et al [60] conducted a study using olive mill wastewater for the production of biohydrogen in a two-stage process, like that shown in Figure 2.5. The authors observed a three fold increase in hydrogen production when compared to photo-fermentation alone and COD conversion efficiency of ~55%. It was postulated high COD concentrations may have had an inhibitory effect and COD removal could be increased by diluting the wastewater. In similar work, Kim et al [36] achieved almost 70% conversion efficiency in a two-step process that utilized C. butyricum and R. sphaeroides using C. reinhardtii biomass (mainly glucose-starch) as substrate.

Work with co-cultures of both C. butyricum and R. sphaeroides showed only a slight increase in the hydrogen yield when compared with production obtained from pure cultures separately [9]; even at high Rhodobacter ratios (~6:1) the bacteria were not able to compete with Clostridium for the substrate. Interactions between the two species could have impeded hydrogen production as both bacteria concentrated their efforts on increasing their respective cell concentration. This work suggests that the two step approach, where the two species are separated, shows more promise in achieving economical hydrogen production yields in large-scale applications.
2.5  *Concluding remarks*

Biological hydrogen production has two main advantages over the traditional hydrogen generating methods: it produces less toxic and green-house gases, and it allows for the simultaneous disposal of human wastes. Anaerobic and photosynthetic degradation techniques capitalize on these advantages by utilizing micro-organisms to convert wastes into hydrogen, though process conditions such as pH and nutrient availability have a strong impact on both hydrogen yield and the rate of gas production. Studies utilizing waste materials, including industrial wastewater, as substrates have shown promise, though more work is still needed on increasing hydrogen production. Many speculate that yields in the vicinity of 60–80% [2] are needed before biological hydrogen production is economically feasible in large applications; the two-step (combined) approach proves to be a promising way to meet this conversion target.
3 Materials and Methods

3.1 Overall approach
The overall approach of the project was designed to:

1) Study the effect of initial pH and glucose concentration (over a pertinent range) on the hydrogen yield and production rate in a synthetic wastewater media on a batch scale;
2) Identify and quantify main soluble products of the fermentation process;
3) Investigate the effect of some key nutrients on the hydrogen evolution (yield and rate of production);
4) Conduct a preliminary assessment of the suitability of raw industrial wastewater as a potential feedstock for biohydrogen production.

Batch experiments were carried out in replicates (duplicate or triplicate) as indicated in the following sections, in order to address the above items.

3.2 Bacterial cultivation

Pure culture of Clostridium beijerinckii was purchased from ATCC (#8260) and used for the duration of the study. The bacterium was pre-cultured in the recommended nutrient broth (Difco ™ Reinforced Clostridial Medium) at 30°C in serum bottles inside a dark incubator shaker. The bacteria were transferred 2-3 times, using Hungate technique, and cultivated for 12 hours (till stationary phase) between each transfer prior to use in the experiments in order to ensure a healthy and active culture population (see section . 3.4.1).

3.3 Experimental procedures

3.3.1 Hydrogen production using standard synthetic wastewater

In order to investigate the hydrogen production potential of C. beijerinckii, batch hydrogen production experiments were carried out in 100 mL serum bottles (Sigma-Aldrich) with a working volume of 50 mL and glucose (CAS# 50-99-7) as
the main carbon source. The media contained a prescribed amount of glucose (see section 3.3.2) as well as essential growth nutrients [9] as shown in Table 3.1 below.

### Table 3.1: Composition of the standard synthetic wastewater solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/L)</th>
<th>Supplier</th>
<th>CAS #</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>850</td>
<td>Sigma</td>
<td>7778-77-0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>750</td>
<td>Sigma</td>
<td>7758-11-4</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>3</td>
<td>Fisher</td>
<td>7782-63-0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200</td>
<td>Sigma</td>
<td>10034-99-8</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>1</td>
<td>Fluka</td>
<td>10102-40-6</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1</td>
<td>Sigma</td>
<td>7446-20-0</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>2</td>
<td>SAFC</td>
<td>10034-96-5</td>
</tr>
<tr>
<td>Cu(NO₃)₂·3H₂O</td>
<td>0.1</td>
<td>ScholAR Chemistry</td>
<td>10031-43-3</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>1</td>
<td>Sigma</td>
<td>10035-04-8</td>
</tr>
<tr>
<td>EDTA</td>
<td>2</td>
<td>Fisher</td>
<td>60-00-4</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>12</td>
<td>Sigma</td>
<td>7782-63-0</td>
</tr>
<tr>
<td>Thiamine</td>
<td>4</td>
<td>MP Biomedicals Inc</td>
<td>67-03-8</td>
</tr>
<tr>
<td>Biotin</td>
<td>3</td>
<td>Sigma</td>
<td>58-85-5</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>5</td>
<td>Sigma</td>
<td>150-13-0</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>6.5</td>
<td>Fluka</td>
<td>98-92-0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>420</td>
<td>Sigma</td>
<td>6106-04-3</td>
</tr>
<tr>
<td>Resazurin</td>
<td>1</td>
<td>Aldrich</td>
<td>62758-13-8</td>
</tr>
</tbody>
</table>

In order to prevent pH decrease due to organic acid accumulation during bacterial metabolism, a 0.1 M phosphate buffer was also added to the media. The initial pH was adjusted to a desired value using 5N NaOH or 5N HCl. The media was boiled under a condenser set-up for 30 minutes to activate the oxidation indicator and drive off dissolved oxygen from the solution. Empty serum bottles were placed in a water/ice bath and continuously flushed with oxygen-free argon gas (CAS # 7440-37-1). The media was then dispensed into the serum bottles and cooled under the flow of argon for 10 minutes. Once cool, the bottles were capped with a butyl rubber stopper, sealed with an aluminium crimp and sterilized in an autoclave. A detailed media preparation technique can be seen in Appendix A.
In order to remove any residual oxygen in the media, prior to inoculation a reducing agent, Na$_2$S.9H$_2$O (CAS # 1313-84-4) was added at a 0.025% (w/w) concentration. All bottles were inoculated, using Hungate technique, with 3% (v/v) C. beijerinckii in the stationary phase and incubated in an orbital shaker (New Brunswick Scientific) at 30°C ± 1°C and rotational speed of 180 rpm. Throughout each experiment biogas and hydrogen concentration measurements were conducted at regular time intervals (within each batch) after an initial acclimatization period of ~12 hours. Experiments were deemed complete when no biogas production was observed for at least 24 hours.

3.3.1.1 Varying key nutrient concentrations

When working with pure anaerobic bacteria, special consideration must be given to the make up of the growth media. Availability of key nutrients such as metals, vitamins and inorganic ions, is crucial for proper bacterial growth and, in the case of clostridia, hydrogen production. For this reason, it is possible that some industrial wastewater streams would need to be supplemented with certain compounds in order to make them suitable feedstocks for biohydrogen production.

Chemical addition to waste streams is not unusual (ex. addition of flocculating agents in wastewater treatment) especially when it results in a desirable outcome such as production of a gas fuel, further downstream. Some of the nutrients in Table 3.1, however, are costly and the need for their addition could cause the process to be economically unfeasible.

In order to investigate the effect of key nutrient concentration on hydrogen production, a number of experiments were conducted on the four most expensive nutrients: biotin, K$_2$HPO$_4$, KH$_2$PO$_4$ and MgSO$_4$.7H$_2$O, as based on Figure 3.1. The value on the y-axis was calculated by dividing the cost of the compound by the concentration quoted in Table 3.1. The cost of each compound was taken as the price paid by the researcher for the quantity needed for laboratory-scale work, and was not normalized with respect to bulk orders, hence the cost per liter value should serve as a rough/relative guideline only.
Figure 3.1: Cost of nutrients per liter of media. The price is quoted in Canadian dollars.

Synthetic wastewater was prepared as described in section 3.3.1, but the concentration of the nutrients was varied (one at a time for biotin and MgSO₄·7H₂O) as shown in Table 3.2 below.

Table 3.2: Variation of key nutrients (biotin, K₂HPO₄, KH₂PO₄ and MgSO₄·7H₂O) in the synthetic wastewater.

<table>
<thead>
<tr>
<th>Trial Number</th>
<th>[Biotin] (mg/L)</th>
<th>[KH₂PO₄] (mg/L)</th>
<th>[K₂HPO₄] (mg/L)</th>
<th>[MgSO₄·7H₂O] (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0</td>
<td>850</td>
<td>750</td>
<td>200</td>
</tr>
<tr>
<td>B2</td>
<td>1.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>2.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>K2</td>
<td></td>
<td>167</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>K3</td>
<td></td>
<td>333</td>
<td>333</td>
<td></td>
</tr>
<tr>
<td>K4</td>
<td></td>
<td>500</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>3</td>
<td>850</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td></td>
<td></td>
<td>33</td>
</tr>
<tr>
<td>M3</td>
<td></td>
<td></td>
<td></td>
<td>67</td>
</tr>
<tr>
<td>M4</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>
For experiments K1-K4 the two potassium phosphate salts were adjusted simultaneously and the mass ratio of 1 (mg KH$_2$PO$_4$/mg K$_2$HPO$_4$) was maintained. In total twelve experiments were carried out, each one in triplicate to ensure reproducibility. For all experiments, the glucose concentration and pH were 3 g COD/L and 6.1 respectively.

3.3.1.2 Varying iron (Fe$^{2+}$) concentration

In saccharolytic clostridia, such as C. beijerinckii, hydrogen evolution occurs via the hydrogenase enzyme as the cells dispose of excess electrons resulting from the breakdown of carbohydrates. An important constituent of the hydrogenase is iron and previous studies by Lee et al [59] have suggested that increasing the Fe$^{2+}$ concentration in the external environment results in an increase in both the yield and the specific hydrogen production rate.

In order to investigate the effect of Fe$^{2+}$ concentration on the hydrogen evolution and production rate, five separate experiments were conducted. Synthetic wastewater was prepared as described in section 3.3.1 but the concentration of FeSO$_4$.7H$_2$O was set to 0, 0.012, 0.05, 0.075 and 0.12 g/L, which translates to an Fe$^{2+}$ concentration of 0, 2.4, 10, 15 and 25 mg/L (Fe0, Fe2.4 Fe10, Fe15, Fe25) respectively. For all five experiments, the glucose concentration and pH were 3 g COD/L and 6.1 respectively. Each experiment was done in duplicate to ensure reproducibility.

3.3.2 Factorial design

In order to analyze the effect of initial glucose concentration and initial pH as well as any interactions between the two variables, a fractional factorial design was employed [61]. A nine trial design was constructed as shown in Figure 3.2 to cover the area of interest.
The substrate concentration varied from 1 to 3 g COD/L with the central value of 2 g COD/L and the pH varied from 5.7 to 6.5 with a central value of 6.1. Both of the ranges were based on values previously observed in a number of local industrial wastewater streams (see Appendix C). The pH range was modified from the original design of 4.5 - 6.5 after no growth was observed in the lower end of the range.

3.3.3 Preliminary industrial wastewater experiments

To test the suitability of an industrial wastewater as a potential feedstock for biohydrogen production, wastewater from a local yogurt manufacturer was acquired. A 2L sample was collected in a polypropylene container and stored at 4°C for approximately a week. The initial pH and chemical oxygen demand (COD Reactor, Hach) were measured, both immediately after obtaining the sample and just prior to start of experiments, and the total (TS), suspended (TSS) and dissolved solids (TDS) were determined according to standard testing procedures [62]. The wastewater characteristics are shown in Table 3.3.

Table 3.3: Yogurt wastewater characteristics.

<table>
<thead>
<tr>
<th>TS (g/L)</th>
<th>TSS (g/L)</th>
<th>TDS (g/L)</th>
<th>COD (g/L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prior to storage</td>
<td>Prior to experiment</td>
</tr>
<tr>
<td>4.03</td>
<td>0.53</td>
<td>3.5</td>
<td>4.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>
The wastewater was then divided into three portions, one for each of the planned experiments;

1) Wastewater with pH adjustment;
2) Wastewater with pH adjustment and a buffer addition;
3) Wastewater with pH adjustment, buffer and nutrient addition.

The conditions for each set of experiments are summarized in Table 3.4.

Table 3.4: Conditions for the three sets of experiments with wastewater from a yogurt manufacturer.

<table>
<thead>
<tr>
<th>Experiment # 1</th>
<th>Experiment # 2</th>
<th>Experiment # 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Adjusted to 6.1 with NaOH</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.1 M phosphate</td>
<td>As shown in Table 3.1</td>
</tr>
<tr>
<td>Nutrients Added</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Each batch of wastewater was stirred for 15 minutes after the addition of supplements to ensure a well mixed solution. Next, the wastewater was dispensed into serum bottles in 50 mL portions, the bottles were purged with oxygen-free argon gas for 15 minutes, capped with a butyl rubber stoppers and sealed with aluminum crimps. Prior to inoculation a reducing agent, Na₂S·9H₂O, was added [0.025% (w/w)] to remove residual oxygen from the solution. An additional control experiment containing synthetic wastewater, (see section 3.3.1) glucose concentration of 3 g COD/L and pH of 6.1, was carried out to ensure viability of the inoculum used in the raw wastewater experiments. The control experiments were done in duplicate.

3.4 Analytical techniques and procedures

3.4.1 Bacteria growth monitoring

For all experiments, stationary growth phase of the cultivated *C. beijerinckii* was confirmed by measuring the optical density using a UV – visible spectrophotometer (Cary 100 Bio, Varian). A 1 mL sample of the actively growing culture was extracted from the serum bottle and diluted with 2 mL of deionized water; the sample’s absorbance (optical density) was then measured at a
wavelength of 600 nm against a deionized water blank. A growth curve of *C. beijerinckii* can be seen below.

![Graph showing growth curve for C. beijerinckii.](image)

**Figure 3.3:** Growth curve for *C. beijerinckii*. The error bars represent the standard deviation from duplicates.

### 3.4.2 Biogas measurement and hydrogen concentration analysis

Biogas production was measured periodically throughout the duration of the experiments using 50, 25 and 10 mL glass syringes (B-D Yale) fitted with hypodermic needles as described by Owen et al [63]. The biogas was sampled from the headspace with a 2 mL gas tight syringe (Vici, Baton Rouge) and analyzed for the amount of hydrogen (H$_2$%) using a gas chromatogram (Hewlett-Packard 5890) equipped with a thermal conductivity detector (TCD). The GC was fitted with a stainless steel molecular sieve column (6” x 1/8””) and the injector, oven and detector temperatures were set at 100°C, 80 °C and 100 °C, respectively. Argon was used as the carrier gas with a flow rate of 2 mL/min. The volume of biogas injected for analysis varied from 0.3 to 2 mL. To obtain the total amount of H$_2$ (mL, at 25°C and STP), the peak area from the GC was converted using a calibration curve (see Appendix B).
3.4.3 Soluble metabolite analysis

Following the completion of the biohydrogen experiments, samples were taken from each serum bottle in order to analyze the organic make up of the effluent. Using a disposable syringe fitted with a hypodermic needle, 35 mL of each sample was extracted, using a Hungate technique (Appendix A), from the serum bottles and placed in a 50 mL centrifuge tubes. The samples were then centrifuged (IEC Multi, Thermo) for 10 minutes at 10,000 rpm to settle out the solids. The supernatants were extracted into a clean test tubes and the final pH of the media was measured (Fisher Scientific XL60). Finally, the samples were filtered through a 0.22 μm filter (MCE 25 mm, Fisher) into sterile culture test tubes and stored in a fridge at 4°C for no more than one week. In cases when immediate analysis was not possible, samples were frozen.

On the day of analysis, samples were first diluted ten fold by placing 200 μL of each sample in a micro-centrifuge tube and adding 1.8 mL of deionized water. Next, 0.1 g of silver powder (CAS# 7440-22-4) was added to each tube in order to precipitate any free chlorine from the solution (excess chlorine can corrode sensitive equipment used in the analysis). The samples were then capped and placed on a sample tumbler and tumbled at 1 rpm for one hour. After the precipitation was complete, all samples were centrifuged for 5 minutes at 6600rpm (Micro-centrifuge, Fisher Scientific) to spin down the silver chloride pellets. The supernatant was analyzed for organic acids and alcohols as described in the following sections. A summary of the sample preparation is provided in Figure 3.4.
3.4.3.1 Gas chromatography

Based on the metabolic pathway of C. *beijerinckii*, simple organic acids and alcohols were expected as the main soluble metabolites (see Figure 2.4). As a result, gas chromatogram equipped with a flame ionization detector (Hewlett-Packard 5890) was used to identify and quantify most of the liquid products. The GC was fitted with a Stabilwax column (30 m, 0.32 mmID, 0.25 µm film thickness) and the injector, oven and detector temperatures were set at 45°C, 55 °C and 180 °C, respectively; the oven ramp rate was set at 5.0°C/min. Helium (CAS # 7440-59-7 was used as the carrier gas with a flow rate of 30 mL/min. The pre-treated sample (see section 3.4.3) was extracted using a 10 µL glass syringe (Hamilton) and 2 µL were injected into the GC for analysis.
3.4.3.2 Ion chromatography

Due to the nature of the GC-FID column, an additional analytical technique was necessary to quantify the acetic acid and formic acid; the two peaks had nearly identical retention times under the given GC-FID parameters. For this reason, ion chromatography (IC) was used to quantify the two compounds. Samples were prepared as described in section 3.4.3 and injected into the ion chromatogram (Metrohm 820) using a disposable syringe fitted with a 0.45 μm filter (PTFE, Fisher Scientific) to remove any particulate matter from the solution. The IC was fitted with a Metrosep A Supp column, set at a temperature of 45°C and 3 mM solution of Na₂CO₃ was used as the eluent at a flowrate of 0.8 mL/min. The volume of sample analyzed was 50 µL and sulphuric acid, 100 mM, was used as the column suppressant/regenerate. To obtain the final concentration of each metabolite (ppm), the peak area from the GC or IC was converted using an appropriate calibration curve (see Appendix B).

3.4.4 Glucose concentration

Initial and final glucose concentrations were analyzed using a glucose (HK) assay kit (Sigma-Aldrich). In a reaction catalyzed by the hexokinase enzyme, glucose reacts with the assay reagent to form NADH which is detected spectrophotometrically at 340 nm (Cary 100 Bio, Varian). The increase in absorbance (against a deionized water blank) is directly proportional to the glucose concentration. The detailed assay kit procedure, as provided by Sigma-Aldrich, is shown in Appendix A.

3.5 Data and statistical analysis

The cumulative hydrogen gas production curves were constructed as previously described [64] by measuring the gas composition in the headspace of the bottle and the total volume of the biogas produced at each time interval, and applying the mass balance equation (eqn 1),
\[ V_{H,j} = V_{H,j-1} + C_{H,j} (V_{G,j} - V_{G,j-1}) + V_H (C_{H,j} - C_{H,j-1}) \]  \hspace{1cm} (1)

where \( V_{H,j} \) and \( V_{H,j-1} \) are cumulative hydrogen gas volumes at the current \((i)\) and previous \((i-1)\) time interval, \( V_{G,j} \) and \( V_{G,j-1} \) are the total biogas volumes at the current and previous time interval, \( C_{H,i} \) and \( C_{H,i-1} \) are the fractions of hydrogen gas in the headspace of the bottle as determined by gas chromatography in the current and previous interval, and \( V_H \) is the total volume of headspace in the bottle.

Each of the cumulative hydrogen production curves was modeled using the modified Gompertz equation (eqn 2),

\[
H(t) = H_{\text{max}} \cdot \exp \left\{ - \exp \left( \frac{R_m \cdot e}{H_{\text{max}}} (\lambda - 1) + 1 \right) \right\} \hspace{1cm} (2)
\]

where \( H(t) \) is the cumulative hydrogen production (mL) during the course of the incubation time, \( t \) (hours), \( H_{\text{max}} \) is the hydrogen production potential (mL), \( R_m \) is the maximum production rate (mL H\(_2\)/h) and \( \lambda \) is the duration of the lag phase (h). This model has been commonly used to describe biological production of various gases such as methane, hydrogen and biogas in a batch set up \([44, 47]\).

The cumulative production curves were fit using Matlab 6.5 by minimizing the sum of square error (SSE). Initial estimates for the parameters \( (H_{\text{max}}, R_m \) and \( \lambda) \) were selected based on visual inspection. The hydrogen production potential was normalized with respect to substrate concentration to give the conversion efficiency, \( P_s \) (mL H\(_2\)/g COD/L); the hydrogen production rate, \( R \) was normalized with respect to working volume and defined as \( R_m/V_{\text{media}} \) (mL H\(_2\)/h-L).

The statistical significance of differences between means of the hydrogen yield at different nutrient concentrations was assessed by using paired t hypothesis tests. The statistical significance was tested at the 95% level and levels of significance \( (p) \) are reported in the cases where significant difference was found.
4 Results and Discussion

4.1 Experimental approach and data reproducibility

The use of serum bottles as batch reactors provided a reliable and flexible method for the study of the effect of initial pH and substrate concentration on the hydrogen yield and rate of production. Replicates within each experiment ensured that the differences observed between the batches were not due to random chance but rather were the result of physiochemical changes occurring due to the manipulation of the independent variables (pH and glucose concentration). For all experiments, the ranges of the standard deviation among the triplicates for the two variables of interest, $P_s$ and $R$, were in the range of 3-15 % and 2-35%, respectively showing good reproducibility. Control experiments containing only glucose affirmed the need for trace nutrients and experiments with nutrient-only synthetic wastewater showed the need for a primary carbon source and confirmed that the hydrogen production observed was due to the breakdown of glucose.

Trial number nine (see Table 4.1) was replicated three times over a three month period, starting with new material each time, to ensure reproducibility of results between batches. To ensure true reproducibility, each of the thirteen trials should have been replicated in the same manner, but this was not done due to time restrictions.

4.2 Cumulative hydrogen production

The cumulative hydrogen production curves for each experiment were modeled as described in section 3.5. For each trial, each of the replicates was fitted with the modified Gompertz equation and the parameters of interest ($R_m$, $H_{max}$, $\lambda$) were calculated by averaging the individual results from the triplicates. The cumulative hydrogen production for the replicates of trial nine (pH: 6.1, [S]: 3 g COD/L) is shown in Figure 4.1. It can be seen from the figure that all data fit the model well ($R^2 > 0.996$ for all curves) and the variation between the replicates
was small. In general, all the experiments showed good reproducibility and the average values for the parameters of interest are shown in Table 4.1.

Figure 4.1: Cumulative hydrogen production curves for trial nine (pH: 6.1, [S]: 3 g COD/L) reported at 25°C and 1 atm. Each curve represents one of the triplicates and in all three cases, $R^2 > 0.996$.

It is important to note that hydrogen production was achieved over the entire range of conditions studied, even at low substrate concentrations. Wastewater collected from various industries from the Montreal area has been shown to have COD in the range of 160 - 2800 mg COD/L (see Appendix C) thus in this study, it was important to establish whether any biogas/hydrogen production by *C. beijerinckii* was feasible within that limit prior to commencing experiments with industrial effluents. The hydrogen production potential ($H_{max}$) was $\sim 7$ mL H$_2$ and no greater than 13 mL H$_2$ for experiments carried out at 1 and 1.5 g COD/L, respectively. Nonetheless, both growth and biogas production were achieved at low organic loadings, confirming the potential use of such effluents.
Table 4.1: Average values for $H_{\text{max}}$, $R_m$ and $\lambda$ for the thirteen batch experiments.

<table>
<thead>
<tr>
<th>COD</th>
<th>pH</th>
<th>Trial</th>
<th>$H_{\text{max}}$ (ml H$_2$)</th>
<th>$R_m$ (ml H$_2$/h)</th>
<th>$\lambda$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Average  STDEV</td>
<td>Average  STDEV</td>
<td>Average  STDEV</td>
</tr>
<tr>
<td>1</td>
<td>6.1</td>
<td>1</td>
<td>6.9  0.6</td>
<td>1.0  0.2</td>
<td>10.1  0.4</td>
</tr>
<tr>
<td>1.5</td>
<td>5.9</td>
<td>2</td>
<td>13   1</td>
<td>2.0  0.5</td>
<td>11.4  1.5</td>
</tr>
<tr>
<td>1.5</td>
<td>6.3</td>
<td>3</td>
<td>12.5  0.8</td>
<td>2.1  0.4</td>
<td>16.9  0.9</td>
</tr>
<tr>
<td>2</td>
<td>5.7</td>
<td>4</td>
<td>11.6  0.7</td>
<td>0.2  0.0</td>
<td>20   4</td>
</tr>
<tr>
<td>2</td>
<td>6.5</td>
<td>5</td>
<td>16   1</td>
<td>2.9  0.6</td>
<td>22.9  0.3</td>
</tr>
<tr>
<td>2</td>
<td>6.1</td>
<td>6</td>
<td>19   1</td>
<td>2.4  0.8</td>
<td>23.2  0.4</td>
</tr>
<tr>
<td>2.5</td>
<td>5.9</td>
<td>7</td>
<td>21.5  0.6</td>
<td>2.8  0.2</td>
<td>14.4  0.4</td>
</tr>
<tr>
<td>2.5</td>
<td>6.3</td>
<td>8</td>
<td>21.5  0.6</td>
<td>3.6  0.2</td>
<td>18.0  0.4</td>
</tr>
<tr>
<td>3</td>
<td>6.1</td>
<td>9</td>
<td>31   2</td>
<td>3.3  0.1</td>
<td>14.5  0.1</td>
</tr>
<tr>
<td>3</td>
<td>6.5</td>
<td>10</td>
<td>22   1</td>
<td>3.3  0.4</td>
<td>26.3  2.1</td>
</tr>
<tr>
<td>3</td>
<td>5.7</td>
<td>11</td>
<td>24   3</td>
<td>1.2  0.4</td>
<td>55   7</td>
</tr>
<tr>
<td>1</td>
<td>6.5</td>
<td>12</td>
<td>5.5  0.3</td>
<td>0.2  0.0</td>
<td>5.5   0.4</td>
</tr>
<tr>
<td>1</td>
<td>5.7</td>
<td>13</td>
<td>5.0  0.4</td>
<td>0.4  0.2</td>
<td>33   7</td>
</tr>
</tbody>
</table>

4.3 Effect of initial pH and substrate concentration

In order to assess the effect of substrate concentration and initial pH on the yield and the production rate of hydrogen, the data obtained from the Gompertz modeling was normalized (see section 3.5) and graphed in two three-dimensional plots. A second-order polynomial regression was conducted in order to interpolate/extrapolate results to cover the entire region of interest as outlined in Figure 3.2. The main objective of the regression was to enhance visual understanding of the types of trends that exist within the matrix. As shall be shown in the following sections, the extrapolated regions turned out to be of particular interest upon visual inspection. Four additional experiments, covering the corner points of the matrix, were carried out in order to validate these initial results. The revised fractional factorial design can be seen in Figure 4.2.
By eliminating extrapolated regions from the design a more concrete conclusion can be made with regards to the location of the area where initial pH and substrate concentration provide the most promising operating conditions. Results based on both fractional factorial designs are presented in the following sections.

4.3.1 Specific hydrogen production potential

The specific hydrogen production potential, or the conversion efficiency, fitted based on the original design, is plotted in Figure 4.3a. It is clear from the data that COD loading and initial pH play a role on the yield. The trends indicate that higher conversion is achieved at higher glucose concentration and a mid-range pH in the vicinity of 6.0; the highest $P_s$ of 10.3 mL H$_2$/g (COD/L) was measured at substrate concentration of 3 g COD/L and pH of 6.1. Both high and low-end pH appears to be unfavourable, but this effect is secondary to that of substrate concentration where the difference between the highest and lowest observed value is almost double.
Figure 4.3: Specific hydrogen production potential 25°C and 1 atm (a) based on the fractional factorial design (9 trials); (b) based on the revisited design (13 trials). The marked points indicate the sum of measured values and the residual of the regression.

When the extra four trials (corner points) were added to the matrix, some changes were observed in the regression of the response variable, $P_s$ (see Figure 4.3b). In general, though, the trends discussed above remain intact and the highest $P_s$ is still found in the same region of the surface as before.

The trends observed in this study are similar to those reported by Chen et al [5] and Van Ginkel et al [6] who saw a rise in the specific hydrogen production potential with an increase in sucrose concentration until a certain maximum. The authors hypothesized that high substrate concentrations become inhibitory to the microorganisms as a result of pH depletion and/or hydrogen partial pressure increase. Conversely, at low substrate concentrations bacteria are thought to utilize the carbon source mainly for biomass growth and not biogas production.

Despite consistency in the trends observed, the maximum $P_s$ seen in this work is significantly lower than that reported elsewhere. Khanal et al [47] and Sung et al [65] reported production potential values of 28 and 89 mL H$_2$/g COD/L respectively, in sucrose degradation with mixed anaerobic inoculum. In both studies, however, the substrate concentration was much higher (>11 g COD/L) than in the work done here. Thus, it is possible that at higher substrate
levels $P_s$ obtained in these experiments could be in the same vicinity as that seen elsewhere. In fact, a linear extrapolation of the observed $P_s$ trend along a constant pH of 6.1 gives a value of approximately 25 mL H$_2$/ g COD/L, thus making the comparison to Khanal’s value reasonable. In order to test this hypothesis, though, experiments with higher glucose concentrations would need to be carried out.

4.3.2 Hydrogen production rate

The hydrogen production rate obtained from the nine-trial data is shown in Figure 4.4a. The data indicates that both initial pH and glucose concentration have noticeable effects on the rate of hydrogen production ($R$), and also that there is interaction between these two parameters. At low substrate loadings, the largest $R$ appears to be located in the middle of the pH range, in the vicinity of 6.1. As the glucose concentration increases, however, the high-end pH values seem to favour greater hydrogen production rates. Low pH gives poor $R$ across the entire substrate concentration span. This is somewhat expected, as similar trends have been reported in other works [46, 47]; low pH tends to have an initial inhibitory effect on the bacteria causing a longer lag phase and lower rate of production.

![Figure 4.4: Hydrogen production rate at 25°C and 1 atm (a) based on the initial fractional factorial design (9 trials); (b) based on the revised design (13 trials). The marked points indicate the sum of measured values and the residual of the regression.](image-url)
Based on Figure 4.4a, the largest $R$ appears to be at high initial pH and substrate concentration, a region of the plot that is mostly extrapolated. As a result, conducting experiments at the corners of the matrix became crucial prior to drawing final conclusions regarding the location of highest hydrogen production rate. The new surface plot can be seen in Figure 4.4b. In general the shape, as well as the scale of the graph, remained unchanged, with slight alterations to the extrapolated areas. The highest $R$ of 71 mL H$_2$/((h*L) was measured at pH 6.3 and substrate loading of 2.5 g COD/L, and similarly high rates were observed in the region around these parameter values. This value is in agreement with what was found in literature; maximum volumetric rates of hydrogen production from pure substrates in batch systems have been reported in the range of $30 – 140$ mL H$_2$/((L*h)) [5, 9, 28, 44].

In practice, any treatment/conversion process strives for high yield and fast rate but often, such as in this case, the most promising operating conditions are different for each variable of interest. When this occurs, a compromise must be made. In this system, out of the two response variables, $P_s$ and $R$, the rate is of more interest since continuous biogas systems operate more optimally at low to mid-range hydraulic retention times [52, 57] meaning that maximum conversion is hardly ever achieved. For this reason, more effort should be devoted to maximizing the rate of hydrogen production with partial sacrifice to the conversion efficiency.

The practicality of biohydrogen production has been evaluated by Levin et al [66]. Based on a 50% conversion efficiency and a 95% hydrogen utilization rate, the authors calculated the size of a bioreactor that would be required to power a 5 kW proton exchange membrane fuel cell (PEMFC) using maximum biohydrogen production rates found in literature. The maximum rate obtained in this study, along with some of those reported in other works, were converted in a similar fashion and the result is shown is Table 4.2. It can be seen from the table that reactor volumes estimated for systems with using pure cultures are larger than those utilizing mixed cultures of anaerobic bacteria as inoculum. The most promising result was obtained in a work with silicone-immobilized sludge by Wu
In practical terms, comparing the size of the reactor obtained from this work to that obtained from the data reported by Wu et al, is equivalent to comparing a large room to the size of a small box, respectively. Nonetheless, when one considers the type of industrial applications where biohydrogen systems are envisioned, 40,000 liter reactors are not unreasonable. Furthermore, the work conducted in this study was done on batch scale and it is possible that higher production rates may be realized once a continuous set-up is in place. Also, cell immobilization may aid in maximizing hydrogen generation. Regardless of the method employed, it is clear that additional work is needed in order to increase the hydrogen production rate.

Table 4.2: Estimated size of bioreactors required to power a 5 kW PEMFC from various fermentative hydrogen production systems.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Max H₂ production rate (L/L/h)</th>
<th>Volume of bioreactor (L)</th>
<th>1 x w x h (m³)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. butyricum</td>
<td>Glucose</td>
<td>0.04</td>
<td>73400</td>
<td>4.2 x 4.2 x 4.2</td>
<td>[28]</td>
</tr>
<tr>
<td>C. beijerinckii</td>
<td>Glucose</td>
<td>0.071</td>
<td>41350</td>
<td>3.5 x 3.5 x 3.5</td>
<td>This study</td>
</tr>
<tr>
<td>C. beijerinckii</td>
<td>Glucose</td>
<td>0.090</td>
<td>32620</td>
<td>3.2 x 3.2 x 3.2</td>
<td>[44]</td>
</tr>
<tr>
<td>C. beijerinckii</td>
<td>Glucose</td>
<td>0.145</td>
<td>20250</td>
<td>2.2 x 2.2 x 2.2</td>
<td>[46]</td>
</tr>
<tr>
<td>C. beijerinckii</td>
<td>Sucrose</td>
<td>0.155</td>
<td>28305</td>
<td>3 x 3 x 3</td>
<td>[53]</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Sucrose</td>
<td>0.105</td>
<td>28305</td>
<td>3 x 3 x 3</td>
<td>[53]</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Glucose</td>
<td>0.28</td>
<td>10485</td>
<td>2.2 x 2.2 x 2.2</td>
<td>[52]</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>Sucrose</td>
<td>15.09</td>
<td>195</td>
<td>0.6 x 0.6 x 0.6</td>
<td>[67]</td>
</tr>
</tbody>
</table>

4.4 Soluble metabolites

The composition of the liquid media, following the completion of biogas production, was of significant interest. The reasons for this were two-fold: 1) characterization of the liquid allows for a better understanding of bacterial metabolism and potentially how different parameters (ex. pH and initial substrate concentration) can cause a shift in the expected metabolic pathway, and 2) the knowledge of the organic make up of the effluent resulting from the anaerobic fermentation is essential to the design of a two-step hydrogen production process – the ultimate goal of the biohydrogen research program in chemical engineering at McGill University.
In total, seven different soluble products were identified; acetate, formate, butyrate, ethanol, propanol, butanol and propanoic acid, though butanol and propanol were only present in some of the trials. In all trials, no glucose was detected in the media upon completion of the experiments. A detailed concentration break down for each trial is shown in Appendix D and a summarized version of the most significant products is shown in Figure 4.5. It can be seen from the figure that butyrate (14\% to 63\%), formate (10\% to 45\%) and ethanol (16\% to 40\%) are the main liquid by-products, with butyrate being the prominent metabolite in most of the experiments. Figure 4.5 also shows the specific hydrogen production potential plotted on a secondary y-axis. From the figure, there appears to be no obvious relationship between the hydrogen yield and products concentration. Similar results were observed when the rate, \( R \), was plotted on the secondary y-axis as shown in Figure 4.6. To further test for correlation between the soluble products and the two variables of interest, plots of \( R \) and \( P_s \) as a function of each metabolite concentration were generated (see Appendix D); in all cases, no correlation was observed.

Figure 4.5: Concentration of prominent soluble products, ethanol, formate and butyrate and specific hydrogen production potential for each experiment. Error bars represent standard deviation from triplicates.
From a hydrogen production perspective, acetate and butyrate are the desirable by-products since hydrogen generation occurs via those reactions. Presence of formate, butyrate, ethanol, propanol, butanol and propanoic acid during anaerobic fermentation by clostridia has been widely reported [36, 41, 49] and is in accordance with the bacterial metabolism as shown in Figure 2.4. High concentrations of the by-products are in agreement with the low $P_s$ values that were observed in all experiments since these metabolites represent hydrogen that has not been released as gas. Presence of ethanol is particularly undesirable due to its toxic effect on bacteria.

It is difficult to know whether the production of ethanol was happening simultaneously with hydrogen generation or if there was a shift in the metabolism at some point during the experiment. Lay et al [48] has indicated that a shift from H$_2$/VFA production to solventogenesis occurs around pH 5.6, but no significant pH decrease was observed in any of the experiments. Other authors suggest that alcohol production occurs once the bacteria enter the stationary growth phase [40, 41], while still other works attribute the shift to increasing hydrogen partial pressure [6, 43]. In order to gain a better understanding of the metabolic activity
occurring in the system additional experiments are required; monitoring the composition of the liquid media throughout the duration of the experiment is suggested. This information could help discover not only the cause of the metabolic shift and hence possible methods for steering the systems away from solventogenesis, but more importantly identify a performance variable (ex. formate concentration) that could be used in operating a continuous biohydrogen production process.

4.5  Effects of media composition adjustments

4.5.1  Biotin, magnesium sulphate and potassium phosphate salts

Experiments varying the concentration of key nutrients, biotin, MgSO$_4$.7H$_2$O, K$_2$HPO$_4$ and KH$_2$PO$_4$ were carried out in order to investigate the potential of decreasing the amount of these compounds in the standard synthetic wastewater (SSW) solution (as described in section 3.3.1). The results for hydrogen yield under various concentrations are shown in Figure 4.7, Figure 4.8 and Figure 4.9 for biotin, magnesium sulphate and potassium phosphate salts, respectively. For biotin, the yield obtained at 3 mg/L was significantly greater than that seen at 0 mg/L ($p = 0.022$) and 4 mg/L ($p = 0.023$). No significant differences in the H$_2$ yield were observed in the range of 1.3-3 mg/L; comparing 3 to 1.3 mg/L ($p = 0.06$), 3 to 2.7 mg/L ($p = 0.13$) and 2.7 to 1.3 mg/L ($p = 0.27$). Clearly, presence of biotin in the solution is necessary though it appears that the concentration can be reduced without significantly affecting the yield.

Similarly, in the case of magnesium phosphate, the yield obtained at 0.2 g/L was significantly greater than that obtained at 0 g/L ($p = 0.0014$); no significant difference was observed between results obtained at 0.2 g/L with SSW and those at 0.1 or 0.03 g/L. Results observed at 0.07 g/L were, however, significantly lower when compared to those obtained with the SSW ($p = 0.037$) though, it is believed, that this difference is due to the large variability between triplicates and the significance would likely diminish with an increased number of sample points, especially since the yields at both 0.03 and 0.1 g/L were not
different from that seen at 0.07 g/L \((p = 0.13 \text{ and } p = 0.18, \text{ respectively})\). It is clear from the results that magnesium is necessary in order to obtain biogas production but once more it appears that the concentration could be reduced without much compromise to the yield. Nonetheless, for both biotin and magnesium phosphate, repeated experiments are recommended in order to confirm the significance of the differences and establish an appropriate/necessary concentration of these substances.

![Graph showing hydrogen yield at different biotin concentrations](image)

**Figure 4.7: Hydrogen yield at different biotin concentrations. The error bars represent standard deviation form triplicates.**

In the case of the potassium salts no significant difference in the yield was observed in the concentration range studied, as seen in Figure 4.9. This result alone, however, does not warrant the elimination of these nutrients from the solution; it is also important to examine the effect of nutrient concentration changes on the rate of hydrogen gas production. Though not shown here, longer lag phases and slower overall rates of biogas production were observed during the experiments where nutrient concentration was reduced. For this reason, repeated trials examining the yield, rate of hydrogen gas production as well as lag phase simultaneously are recommended.
Figure 4.8: Hydrogen yield at different MgSO$_4$.7H$_2$O concentrations. The error bars represent standard deviation from triplicates.

Figure 4.9: Hydrogen yield at different K$_2$HPO$_4$ concentrations. The error bars represent standard deviation from triplicates.
4.5.2 Iron (Fe$^{2+}$) concentration

Experiments with varying iron concentration were carried out in order to investigate the effect of increasing Fe$^{2+}$ on the hydrogen yield and production rate. The results for the specific hydrogen potential from the four trials, along with the result obtained with the synthetic wastewater are shown in Figure 4.10. Previous works with mixed cultures of anaerobic bacteria, have shown that an increase of Fe$^{2+}$ in the external environment causes an increase in both the yield and rate of hydrogen production [42, 59]. Lee et al [59] observed maximum hydrogen yield on a sucrose substrate at a concentration of ~ 350 mg Fe$^{2+}$/L; concentrations above this value resulted in a decrease of gas production. Yang and Shen [42] saw similar trends in their work but reported the maximum hydrogen yield at an iron concentration of 55 mg Fe$^{2+}$/L, above which there appeared to be a plateau effect. These results however, are in contrast to what was observed in this study for the concentration range of 0 – 25 mg Fe$^{2+}$/L; the highest $P_s$ of 10.3 mL H$_2$/(g COD/L) was observed under standard synthetic wastewater condition with an iron concentration of 2.4 mg Fe$^{2+}$/L. In all other experiments, the maximum production potential was in the range of 0.7 – 4.7 mL H$_2$/g COD as shown in Figure 4.10. The need for iron in the solution was confirmed by the poor result obtained with Fe0.

The trend seen in Figure 4.10 is puzzling and difficult to account for; it is hypothesized that at a concentration of 10 mg Fe$^{2+}$/L and greater the iron had an inhibitory effect on the bacteria. Though inhibition was reported at higher Fe$^{2+}$ values by others [42, 59], the source of iron and the initial pH were different than those studied here. It might be possible that, under the conditions studied, higher concentrations of iron generated more/different metal complexes (with various ligands), which in turn led to a decrease in the available iron and/or ligand concentration in the external environment. Nonetheless, the result witnessed here is somewhat novel and warrants further investigation; experiments in the 0 to 10 mg Fe$^{2+}$/L range are suggested to test for inhibitory effects.
In addition to the surprising $P_s$ trend observed above, samples Fe10, Fe15 and Fe25 displayed an unusual cumulative hydrogen production profile as shown in Figure 4.10. It can be seen that none of the trials fit the modified Gompertz model equation, but rather displayed a double effect with two plateau and two exponential regions. Due to this anomaly, it was not possible to determine the maximum rate of hydrogen production ($R$). Time before maximum $H_2$ potential was achieved was extremely long and in the case of sample Fe 15, it took over two weeks for production to cease. Additional work is necessary to account for this double-effect behaviour and slow hydrogen evolution.

Figure 4.10: Specific hydrogen production potential for various Fe$^{2+}$ concentrations (pH=6.1, COD=3). The error bars represent standard deviation from duplicates or triplicates.
4.6 Preliminary wastewater experiments

The cumulative hydrogen production from the preliminary experiments with yogurt-wastewater is shown in Figure 4.12. In the first experiment, only the pH of the wastewater was adjusted prior to inoculation and the results are presented in Figure 4.12a. It can be seen that in all three replicates the cumulative hydrogen production is very low (max ~ 3 mL) and not always increasing. Among the triplicates, the decrease in the cumulative H₂ occurred at different times and to a varying degree. In the second experiment where the initial pH was adjusted to 6.1 and a phosphate buffer was added to the wastewater, decrease in the cumulative hydrogen production for all replicates was again observed (Figure 4.12b). Maximum net volume of hydrogen obtained was once more dismal (~4
mL) and the addition of the buffer to the wastewater appears to have had no effect on the performance of the bacteria.

Finally, in the last experiment, in addition to pH adjustment and buffer addition, the wastewater was supplemented with trace nutrients (see Table 3.1). The results for the triplicates can be seen in Figure 4.12c. In two of the replicates hardly any net hydrogen production was observed, however, trial 3A showed an overall positive cumulative hydrogen production profile yielding a total net volume of hydrogen of ~6 mL, this however is still much lower than what was obtained in synthetic wastewater (WW) trials.

The low yield of hydrogen in all three wastewater experiments can be attributed to the nature of the COD content. Although the COD of the wastewater was 4 g COD/L, in the synthetic WW experiments COD was provided in the form of pure glucose. The industrial effluent was obtained from a yogurt manufacturer, meaning that the organic content was most likely composed mainly of proteins. In fact, according to the assay test, the glucose content of the wastewater was below the detection limit. Saccharolytic bacteria are known to preferentially degrade carbohydrates and as such the low hydrogen production may be the result of the proteins being too complex for the bacteria to biodegrade.

The fluctuating cumulative hydrogen production seen in all the trials indicates that other bacteria were most likely present in the wastewater. This is further confirmed by the drop in pH (from 9.32 to 5.32) Studies using mixed culture of anaerobic bacteria for hydrogen production have linked decreases in hydrogen concentrations to the presence of methanogens [64] as these microbes consume hydrogen, to produce methane gas. Although methanogens are strict anaerobes, they are capable of sustaining oxygen stresses for long periods of time. Therefore, it is highly likely that the irregular shape of the cumulative hydrogen curves seen in Figure 4.12 is a result of continuous hydrogen production and consumption occurring simultaneously. Interestingly batch 3A was the only experiment where no decrease in cumulative H₂ production was observed. It is important to note, however, that that does not necessarily mean that no hydrogen consumption was taking place since data shown is really the net volume (H₂
produced – H₂ consumed) of hydrogen as a function of time and if H₂ produced > H₂ consumed at the measured times, the shape of the curve would still hold true. As a result, it is difficult to assess the ‘total’ amount of hydrogen produced in each trial.

Little difference was observed in the lag phase between the first two sets of experiments with first hydrogen detection occurring at approximately the 25 hour mark. When trace nutrient were added to the wastewater, however, the lag phase more than doubled. It is believed that the added nutrients may have been used by other microorganisms allowing them to flourish over the desired bacterium. Presently, it is difficult to account for the discrepancy in the results between batches 3A – 3C. It is clear from the preliminary results that the wastewater should be treated in some manner (pH reduction, sterilization, etc.) prior to use, in order to eliminate any other microorganisms from the system and test the wastewater’s suitability as substrate for biohydrogen production. Alternatively, a full analysis of the biogas sample could provide answers about potential presence of other microbes such as methanogens. Finally, a full characterization of the initial wastewater (type of COD, metal concentration, etc.) is necessary to better understand the initial system. Based on the results obtained here, it is apparent that the yogurt-wastewater cannot be used for biohydrogen production ‘as-is’ and much research is still needed to assess the full potential of this wastewater as a feedstock for fermentative hydrogen production.
Figure 4.12: Cumulative hydrogen production curves for wastewater experiments reported at 25°C and 1 atm; (a) pH adjusted, (b) pH adjusted and buffer added, (c) pH adjusted, buffer and trace nutrient added. In each case, trials A-C indicate replicates.
5 Conclusions

This research has demonstrated that substrate concentration (glucose) and initial pH have an effect on both the hydrogen production potential and rate of hydrogen production for a pure anaerobic bacterium of *C. beijerinckii*. Considering the specific objectives and hypotheses of the work the following conclusions can be drawn:

1) **Greatest conversion efficiency is achieved at high glucose concentration and mid-range pH.** Increasing substrate concentration gives rise to increasing conversion efficiency. In the range studied, highest $P_s$ of 10.3 mL H$_2$/g (COD/L) was observed at substrate concentration and pH of 3 g COD/L and 6.1, respectively. Trends indicate that both high and low-end pH is unfavourable.

2) **Highest rate of hydrogen production occurs at high pH and high glucose concentration.** Increasing both the COD and pH gives results in increasing rate of hydrogen production. In the range studied, highest $R$ of 71 mL H$_2$/h*L was achieved at pH 6.3 and substrate loading of 2.5 g COD/L. Low pH gives poor $R$ across the entire substrate concentration range.

3) **Volatile fatty acids and alcohols are the main soluble products resulting from the fermentation process.** In all experiments, butyrate (14% to 63%) formate (10% to 45%) and ethanol (16% to 40%) were the main soluble metabolites with pronanoic acid (<20%), propanol (<8%), acetate (<5%), and butanol (>1%) present in smaller quantities. No correlation was observed between final metabolite concentration and hydrogen yield or rate of production.

4) **Complete absence of micronutrients biotin and MgSO$_4$·7H$_2$O from the synthetic wastewater solution causes a significant decrease in the hydrogen yield.** On the other hand, decrease in both biotin and magnesium sulphate concentrations (to within the ranges 1.3 – 3 mg/L and 0.03 – 0.1 g/L, respectively) showed no significant effect on the hydrogen yield when
compared standard synthetic wastewater. Change in K₂HSO₄ and KH₂SO₄ had no impact on the hydrogen yield in the range studied.

5) **Increasing the concentration of FeSO₄.7H₂O from 12 mg/L to 50, 75 or 120 mg/L has a negative effect on both the overall rate of hydrogen gas production and the conversion efficiency.** In the concentration range of 0 – 25 mg Fe²⁺/L; the highest $P_s$ of 10.3 mL H₂/(g COD/L) was observed under standard synthetic wastewater condition with an iron concentration of 2.4 mg Fe²⁺/L. The cumulative hydrogen production profiles for iron experiments Fe10, Fe15 and Fe25 were atypical and the time to maximum hydrogen potential was >200 hours; nearly four times greater than that observed at Fe2.4.

6) **The use of raw effluent obtained from a yogurt manufacturer results in poor biohydrogen production.** Nutrient and buffer addition, did not significantly increase the biohydrogen production and the highest $P_s$ was only 1.4 mL H₂/(g COD/L), nearly ten times less than what was achieved with synthetic wastewater.
6 Recommendations

Future research needs pertaining to fermentative biohydrogen production from wastewater as identified in this work are:

1) To test the observed $P_s$ and $R$ trends over a greater range of substrate concentration and pH to see if the behaviour observed is in accordance with that seen in this study.
2) To monitor the composition of the liquid media throughout the duration of the experiment in all future tests in order to gain understanding of the metabolic shift and hence possible methods for steering the systems away from solventogenesis and identify a performance variable that could be used in operating a continuous biohydrogen production process.
3) To test for iron inhibition effects by varying the FeSO$_4$.7H$_2$O concentration between 2.4 and 10 mg Fe$^{2+}$/L in the synthetic wastewater solution.
4) To repeat experiments with the industrial yogurt effluent, this time characterizing the wastewater fully prior to use and eliminating any undesired micro-organisms either through initial pH reduction or sterilization.
5) To conduct continuous experiments, complete with pH control in order to simulate more realistic process operating conditions and to study the effect of other pertinent variables, such as hydraulic retention time and hydrogen partial pressure, on biohydrogen production.
7 References


Appendix A: Procedures

Synthetic media preparation

All micronutrients were weighed out on a microbalance and dissolved in de-ionized water. Glucose and phosphate buffer were added as per experimental design and the media was stirred and boiled under a condenser set up for 20 – 45 minutes to activate the redox indicator (solution turns pale pink) and drive off oxygen from the solution. 50 mL of the solution was then transferred (using a 50 mL glass pipette) to each serum bottle under the flow of oxygen-free argon gas. Prior to the transfer the bottles were placed inside a cooling bath (cold water + ice) to help speed up the cooling process. The media inside the bottles was cooled for 10 – 15 minutes, after which the bottles were capped with thick butyl rubber stoppers and sealed with an aluminum crimp. All media-containing bottles were placed in turkey-pan container which was filled with water, approximately 1 inch in depth, and placed inside the autoclave for sterilization (15 minutes at 121°C and 15 psi).

After sterilization was complete, the bottles were removed from the autoclave and allowed to cool to room temperature. Each bottle was then reduced using Na₂S.9H₂O, inoculated with an actively growing bacterial culture and incubated at 30°C±1°C for the duration of the experiment.

Hungate technique

The Hungate technique involves the over pressurization of sealed bottles with an inert, sterile gas prior to any liquid extraction from the bottle. All transfer, inoculation and liquid sampling was conducted using sterile disposable hypodermic needles and syringes which allowed for easy manipulation of the cultures without compromising sample purity. A sample of oxygen-free gas was withdrawn from a sampling bag through a 0.22 μm filter (Fisher Scientific) and
the vessel of interest (i.e. vial, tube or bottle) was overpressurized as shown in Figure A 1. A liquid sample, equal in volume to the sterile gas that was injected, was then withdrawn to equilibrate the pressure inside the vessel.

Figure A 1: Over pressurizing of media with a sterile, O₂-free gas [68].
Glucose Assay

The glucose assay procedure obtained from the manufacturer is shown below.

**Glucose (HK) Assay Kit**

**Product Code**: GAHK 20

**Storage Temperature**: 2–8 ºC

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**Product Information**

**Technical Bulletin**

**Product Description**

Enzymes, as analytical tools, have found widespread use in the food, biochemical, and pharmaceutical industry. Enzymatic methods are specific, reproducible, sensitive, rapid, and therefore, ideal for analytical purposes. Due to the high specificity and sensitivity of enzymes, quantitative assays may be done on crude materials with little or no sample preparation. This kit is for the qualitative, enzymatic determination of glucose in food and other material.

**Principle**

\[
\text{Hexokinase} \
\text{Glucose + ATP} \rightarrow \text{Glucose-6-Phosphate + ADP} \
\text{G6PDH} \
\text{G6P + NAD} \rightarrow \text{6-Phosphogluconate + NADH}
\]

Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose-6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD). In a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

**Components**

1. Glucose (HK) Assay Reagent
   (Product Code: G 5203)
   Reconstitute the vials contents with 20 ml of water. After addition of water, stopper the vial and immediately mix several times by inversion. DO NOT SHAKE.

   Each vial when reconstituted with 20 ml of water contains 1.5 mM NAD, 1.0 mM ATP, 1.0 unit/ml of hexokinase, and 1.0 unit/ml of glucose-6-phosphate dehydrogenase with sodium benzoate and potassium sorbate as preservatives.

   The dry reagent is stored at 2-8 ºC. The reagent should be discarded if the vials contents exhibit caking due to possible moisture penetration, if the vial contents do not dissolve completely upon reconstitution, or if the reconstituted solution appears turbid.

   The reconstituted reagent is stable, in the absence of visible microbial growth for 7 days at 15-20 ºC and for at least 4 weeks at 2-8 ºC. The reagent is not suitable for use if the absorbance of the freshly reconstituted solution measured at 340 nm versus water at the reference is greater than 0.250.

2. Glucose Standard Solution
   (Product Code: G 3265)
   D-Glucose, 1.0 mg/ml in 0.1% benzoic acid. This standard is traceable to an NIST standard and is supplied ready-to-use. It is stable at 2-8 ºC for at least six months. Discard if turbidity develops.

**Equipment Required but Not Provided**

2. Cuvets
3. Pipettes capable of accurately dispensing 10 µl to 1 ml.

**Precautions and Disclaimer**

This product is for R&D use only, not for drug household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

**Storage/Stability**

Store the kit at 2-8 ºC.
Procedure

Sample Preparation:

Liquids - Dilute sample with deionized water to 0.5 - 5 mg of glucose/ml.

Filter or deproteinize solution if necessary to clarify. Solutions that are strongly colored and that have a low glucose concentration should be deproteinized. Carbonated or fermented products must be degassed.

Solids - Weigh out sample to nearest 0.1 mg. Extract sample with deionized water. The solution may be heated (~75°C) to aid extraction. Dilute with deionized water to 0.5 - 5 mg of glucose/ml. Filter or deproteinize solution if necessary, to clarify.

Determination:

Pipe out a volume of solution corresponding to 0.5 - 50 μg of glucose. Repeat assay and vary the sample volume, if necessary, to give an ΔAbs between 0.03 and 1.0.

1. Pipe out the following solutions into the appropriately marked test tubes:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Glucose Assay Reagent (ml)</th>
<th>Sample Volume (ml)</th>
<th>Volume of Deionized Water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Blank</td>
<td>—</td>
<td>Same as for Test</td>
<td>1.0</td>
</tr>
<tr>
<td>Reagent Blank</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Test</td>
<td>1.0</td>
<td>10.200</td>
<td>—</td>
</tr>
</tbody>
</table>

2. Mix tubes and incubate for 15 minutes at room temperature (10-35°C).

3. Measure the absorbance at 340 nm versus deionized water.

Calculations:

The total blank must take into account the contribution to the absorbance of the sample and the glucose assay reagent.

\[ \text{mg glucose/ml} = \frac{(\text{mg total blank} - \text{mg reagent blank}) \times \text{Sample Volume (ml)} \times \text{Conversion Factor for mg to mg}}{100} \]

\[ \text{mg glucose/ml} = \frac{(\Delta \text{Abs} \times \text{SV} \times \text{F})}{100} \]

\[ \Delta \text{Abs} = \frac{\text{Abs} \text{Test} - \text{Abs} \text{Sample Blank}}{\text{Abs} \text{Reagent Blank}} \]

\[ \text{SV} = \text{Sample Volume (ml)} \]

\[ \text{Conversion Factor for mg to mg} = 100 \times \text{Sample Volume (ml)} \]

\[ \text{Sample Volume (ml)} = \frac{\text{Sample Volume (ml)}}{100} \]

\[ \text{Conversion Factor for mg to mg} = \frac{\text{Sample Volume (ml)}}{100} \]

References:


68
Appendix B: Calibration curves

Hydrogen

The hydrogen calibration curve was constructed by injecting known volumes of a 100% hydrogen standard into the GC-FID. The curve is shown below.

![Hydrogen calibration curve](image)

\[ y = 796690x + 39463 \]

\[ R^2 = 0.9976 \]

Figure A 2: Hydrogen calibration curve. The error bars represent the standard deviation from four separate injections.

Soluble products

Calibration curves for the prominent soluble metabolites (ethanol, propanol, butanol, propanoic acid, butyric acid, and formate) were constructed using analytical standard solutions shown in the table below. The calibration curve for acetic acid was obtained from the IC software and hence the curve is not shown.
Table A 1: Purity of standards used in the construction of soluble products calibration curves.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purity</th>
<th>Supplier</th>
<th>CAS#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>99%</td>
<td>Sigma-Aldrich</td>
<td>64-17-5</td>
</tr>
<tr>
<td>Propanol</td>
<td>98%</td>
<td>Sigma-Aldrich</td>
<td>71-23-8</td>
</tr>
<tr>
<td>Butanol</td>
<td>95%</td>
<td>Sigma-Aldrich</td>
<td>71-36-3</td>
</tr>
<tr>
<td>Propanoic acid</td>
<td>98.9%</td>
<td>Sigma-Aldrich</td>
<td>79-09-4</td>
</tr>
<tr>
<td>Formic acid</td>
<td>88%</td>
<td>Sigma-Aldrich</td>
<td>64-18-6</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>&gt;98%</td>
<td>Sigma-Aldrich</td>
<td>107-92-6</td>
</tr>
</tbody>
</table>

Due to the volatile nature of the compounds of interest, the calibration standards were prepared using a gravimetric technique inside headspace vials sealed with a silicon septum. Water was first weighed inside a previously zeroed 10 mL headspace vial. The vial was then closed with a silicone septa cap, and re-weighed on the balance. A small volume of the compound of interest (from table above) was added to the vial using a disposable syringe and needle and the vial was re-weighed. Serial dilutions were then made using the same procedure to provide standards with concentrations spanning the range of interest. Each standard was injected into the GC-FID to generate the desired calibration curves which are shown below.

\[
y = 280.69x - 5217.9 \\
R^2 = 0.996
\]

Figure A 3: Ethanol calibration curve. The error bars represent the standard deviation from four independent injections.
Figure A 4: Propanol calibration curve. The error bars represent the standard deviation from four independent injections.

Figure A 5: Butanol calibration curve. The error bars represent the standard deviation from four independent injections.
**Figure A 6:** Propanoic acid calibration curve. The error bars represent the standard deviation from four independent injections.

\[ y = 150.41x + 523.43 \]
\[ R^2 = 0.9967 \]

**Figure A 7:** Formic acid calibration curve. The error bars represent the standard deviation from four independent injections.

\[ y = 189.77x - 725 \]
\[ R^2 = 0.9931 \]
Figure A 8: Butyric acid calibration curve. The error bars represent the standard deviation from four independent injections.
Appendix C: Wastewater characteristics

Wastewater was collected from various industries in the Montreal area in the summer of 2006, and analyzed as shown in the table below. The experimental design used in this study was then based on the COD values observed in such effluents.

Table A 2: Wastewater properties from effluents obtained from industries in the Montreal area.

<table>
<thead>
<tr>
<th>Wastewater Properties</th>
<th>Industry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soft Drink</td>
</tr>
<tr>
<td></td>
<td>Food</td>
</tr>
<tr>
<td></td>
<td>Sugar (Collector 18)</td>
</tr>
<tr>
<td></td>
<td>Sugar (Final Effluent)</td>
</tr>
<tr>
<td></td>
<td>Yogurt</td>
</tr>
<tr>
<td></td>
<td>Drug</td>
</tr>
<tr>
<td>pH</td>
<td>6.65</td>
</tr>
<tr>
<td>COD (mg/L)</td>
<td>2400</td>
</tr>
<tr>
<td>Total solids (mg dry/ mL)</td>
<td>1600</td>
</tr>
<tr>
<td>Total dissolved solids (mg dry/ mL)</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>6.61</td>
</tr>
<tr>
<td></td>
<td>6.85</td>
</tr>
<tr>
<td></td>
<td>890</td>
</tr>
<tr>
<td></td>
<td>850</td>
</tr>
<tr>
<td></td>
<td>690</td>
</tr>
<tr>
<td></td>
<td>4.96</td>
</tr>
<tr>
<td></td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>550</td>
</tr>
<tr>
<td></td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
</tr>
<tr>
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<td>6.6</td>
</tr>
<tr>
<td></td>
<td>8.45</td>
</tr>
<tr>
<td></td>
<td>2800</td>
</tr>
<tr>
<td></td>
<td>2600</td>
</tr>
<tr>
<td></td>
<td>2600</td>
</tr>
<tr>
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<td>6.34</td>
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<td></td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>4.05</td>
</tr>
<tr>
<td></td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>360</td>
</tr>
</tbody>
</table>
Appendix D: Soluble products

Soluble products identified at the end of the hydrogen production for all batch experiments are shown in the table below.

Table A 3: Concentrations of identified soluble products. Standard deviation is from triplicates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Trial number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>241±8</td>
<td>230±30</td>
<td>250±20</td>
<td>260±20</td>
<td>290±80</td>
<td>275±70</td>
<td>235±20</td>
<td>280±20</td>
<td>220±5</td>
<td>290±40</td>
<td>220±20</td>
<td>245±15</td>
<td>210±2</td>
</tr>
<tr>
<td>Propanol</td>
<td></td>
<td>65±10</td>
<td>20±30</td>
<td>40±35</td>
<td>0</td>
<td>30±40</td>
<td>30±40</td>
<td>0</td>
<td>40±35</td>
<td>0</td>
<td>20±30</td>
<td>0</td>
<td>60±2</td>
<td>0</td>
</tr>
<tr>
<td>Butanol</td>
<td></td>
<td>2±3</td>
<td>0</td>
<td>0.5±0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td></td>
<td>370±15</td>
<td>380±5</td>
<td>300±30</td>
<td>510±20</td>
<td>150±45</td>
<td>155±135</td>
<td>100±6</td>
<td>355±2</td>
<td>105±2</td>
<td>150±9</td>
<td>420±10</td>
<td>145±5</td>
<td>380±20</td>
</tr>
<tr>
<td>Acetate</td>
<td></td>
<td>30±2</td>
<td>25±1</td>
<td>35±3</td>
<td>20±2</td>
<td>35±2</td>
<td>25±6</td>
<td>30±15</td>
<td>40±2</td>
<td>35±2</td>
<td>45±2</td>
<td>30±5</td>
<td>30±2</td>
<td>20±1</td>
</tr>
<tr>
<td>Propanoate</td>
<td></td>
<td>180±40</td>
<td>55±4</td>
<td>120±25</td>
<td>0</td>
<td>120±35</td>
<td>70±20</td>
<td>0</td>
<td>85±25</td>
<td>45±7</td>
<td>120±5</td>
<td>55±15</td>
<td>110±8</td>
<td>60±2</td>
</tr>
<tr>
<td>Butyrate</td>
<td></td>
<td>180±150</td>
<td>405±60</td>
<td>250±20</td>
<td>510±85</td>
<td>330±60</td>
<td>135±100</td>
<td>405±60</td>
<td>520±15</td>
<td>700±125</td>
<td>535±80</td>
<td>595±80</td>
<td>95±10</td>
<td>180±15</td>
</tr>
</tbody>
</table>

As a test for correlation between the soluble products and the two variables of interest, plots of \( R \) and \( P_s \) as a function of each metabolite concentration were generated. The plots for ethanol, propanol, butanol, formate, acetate, propanoate and butyrate as a function of specific hydrogen potential and hydrogen production rate are shown below.
Figure A 9: Specific hydrogen production potential and hydrogen production rate as a function of formate and acetate concentration.
Figure A 10: Specific hydrogen production potential and hydrogen production rate as a function of propanoate and butyrate concentration.
Figure A 11: Specific hydrogen production potential and hydrogen production rate as a function of ethanol and propanol concentration.
Figure A 12: Specific hydrogen production potential and hydrogen production rate as a function of butanol concentration.