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Potential production of Hydrogen through dark fermentation, with sludge from Suchdolský Jeník brewery as inoculum.

Diploma Thesis
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Declaration

I declare that the Diploma Thesis “Potential production of Hydrogen through dark fermentation, with sludge from Suchdolský Jeník brewery as inoculum “is my own work and all the sources I cited in it are listed in Bibliography.

Prague, 24/07/2020 Signature ____________________________

Acknowledgements

I would like to express my gratitude and thanks to my supervisor Doc. Ing. Vladimír Hönig, Ph.D. et Ph.D. for all the guidance, to the colleague Jan Jenčik for all the help in the practical work, as well as the members of the chemical department from the faculty of agrobiology, food and natural resources, they were great help and even invited me to have share a Czech lunch with them, and special thanks to the Suchdolský Jeník brewery for providing the samples of their residue.

I would also like to thank my friends who listened to me talk about the thesis and by doing so, helped me realise mistakes I was making, and specially to Faisal Kelani and Claudia Lugo who apart from listening, actively help me with the chemical and mathematical part of the discussion. Finally, I would like to thank my parents for all the emotional and economical support that they gave me, without it, I would not have been able to start and finish this master’s program.

Potential production of Hydrogen through dark fermentation, with sludge from Suchdolský Jeník brewery as inoculate

Summary

Three batch anaerobic reactors were set up using a sample of the residual sludge from Suchdolský Jeník brewery to assess the potential of the sludge to produce hydrogen under anaerobic conditions. The reactors were set at a volume of 600 ml, 35°C and pH of 5,65; glucose was added so this would not be a restricting factor. The produced gas was measured with the water displacement method. The results were 0, 60 and 145 ml, the actual hydrogen gas was calculated using a stoichiometric formula, where for each mole of glucose, 4 moles of hydrogen were produced, this led to the calculation of the efficiencies of the reactors, giving 0%, 1.17% and 2.83%. The low efficiencies suggest a low potential of the sludge as an inoculum for producing hydrogen under anaerobic environment at the set conditions. The low potential could be explained by the possible low concentration of the bacteria of interest in the sludge and unexpected changes in the environmental conditions in the reactors.

Key words: Hydrogen, dark fermentation, batch reactor, *Clostridium*.

Content

1 Introduction

The world is facing an increasing demand of energy, causing harm to the environment and endangering the survival of the future generations; therefore, humankind is looking for technologies that can minimize these negative impacts (Abánades, 2012). Hydrogen as a source of energy has been considered since the decade of 1970, it can be used in combustion engines or in fuel cells. Its combustion does not produce any CO2 and the NOx that can be produced can be controlled with the air supply and it has the higher energy content in a unit weight of all the known fuels. Its disadvantages are the difficulty to store it, and the fact that its production is expensive and inefficient (Boboescu et al., 2014; Obergruber et al., 2018).

There are various ways to produce hydrogen, such as electrolysis, thermal decomposition of sulphate, gasification of coal, steam reforming, partial coal oxidation, biomass pyrolysis, thermolysis and biochemical processes (Obergruber et al., 2018). From these processes, the biological ones consume the least energy. These biological processes are divided between light and non-light dependant, the light dependant are bio photolysis, photo fermentation, dark fermentation and microbial electrolysis cell (Ghimire et al., 2015).

According to (Abánades, 2012), 96% of the hydrogen produced worldwide comes from fossil fuels, which implies greenhouse emissions among other problems. This vast percentage is divided as it follows: 48% from natural gas team reforming, 30% from naphtha/oil reforming and 18% from coal gasification.

Hydrogen production needs to be enforced, especially fossil fuel free methods, according to the European Commission, in 2050, 30% of hydrogen will be produced from coal, within the carbon dioxide free methods the estimate is that nuclear takes around 10% and the rest of renewable sources the remaining percentage (Abánades, 2012).

It is in that last percentage, that dark fermentation takes place. This method is one of the least harmful for the environment, it is among the most cost effective, and it concerns another environmental issue such as industry residues. Authors like (Manyuchi et al., 2018), (Cui et al., 2009) and (Argun et Kargi, 2009) have reported the use of beer industry's residues to produce hydrogen gas via dark fermentation.

2 Hypothesis and Objectives

2.1.Hypothesis

The volume of the synthesis gas will be between 100 and 300 ml.
The synthesis gas produced will have 4 mol of Hydrogen per mol of used glucose as main product, and another sub product, CO2.

2.2.Objective

To assess the potential production of Hydrogen through dark fermentation, with sludge from Suchdolský Jeník brewery as inoculum.

3 Justification

Beer producing industry make a big part of Czech Republic's economy, (Flanders Investment et Prague Trade, 2017) state that the country is the seventh biggest producer in the European Union with an approximated total annual trade of 2,05 billion, which is mostly produced within the country, 98% by the year 2016.

Beer is an alcoholic beverage with a low alcohol content, its production consists in the fermentation of barley wort, sometimes mixed with other grains, the fermentation process is done by Saccharomyces, cerevisiae or carlsbergensis, and hops or its extracts are added (Basanta et al., 2008).

A great quantity of residues is created at the stage of the production of the wort. The wort in the Czech Republic is done using more than one mashing, up to three; this method, also known as decoction, varies from the infusion method in which the mash is heated all at the same time, shorting the whole process, this method is the one used in western countries (Puskarčík, 2013). The sludge formed during the wort production is the product in which there can be a higher presence of microorganisms, including hydrogen producing bacteria.

(Brožová et al., 2018) report that bacteria of the genus *Clostridium* can survive the boiling temperatures of the wort production due to their ability of forming endospores, but due to the bitter hop substances and acidic conditions, the growth of those endospores is inhibited in later stages of the beer production.

(Menz et al., 2009) also state that bacteria are more likely to be present in intermediate products such as the wort sludge, because if some spore forming bacteria resist the boiling temperatures, the presence of ethanol formed by the fermentation done by the yeasts, inhibits the presence of the microorganisms in the finished beer. Contrarily, (Hawthorne et al., 2018) reported that the presence of butyric acid in beer is an indication of the presence of spore forming bacteria such as *Clostridium* in the latest steps of the production of beer, but they attribute it to poor hygiene in brewing plants.

Suchdolský Jeník brewery removes the sludge and the wort by means of centrifugate force in a special vessel named called whirlpool, the temperature of the mixture is 99°C (Česká zemědělská univerzita v Praze, 2019). That is an indication that some microorganisms could survive in the residual sludge, more likely spore forming bacteria, such as *Clostridium*, which according to (Wang et Wan, 2009) are one of the main genus used to produce hydrogen under anaerobic conditions. Such residual sludge, produced in Suchdolský Jeník brewery, is use for feeding the animals of the local farm (Puskarčík, 2013).

The beer industry is known for being a supplier for cow feed, around 20 kg of sludge is produced per 100 litters of produced beer, this residue has a protein content that varies from 14.5 to 33.6%, and has high fibre content which influences in the digestibility of the diet when replacing concentrate feed (Faccenda et al., 2020).

Ruminant feeding is a common use for residual brewery sludge, but there other uses for this residue include, its application as organic fertilizer, as a source of polyphenols and dietary fibre by various methods such as, solvent extraction, adsorption into resins, the use of membranes, ultrasound and microwaves. This residue can also be used to extract valuable components for human nutrition, such as fibre, protein, bioactive compounds and various minerals (Skendi et al., 2018).

(Chudoba et al., 2011) describe examples of anaerobic fermentation of sludge in various places in Central Europe, they report that a Pilsen water treatment plant uses the Pilsner Urquell sludge to produce biogas, they obtain enough energy to cover 100% of heat needs and 74% of electricity needs, respectively.

Anaerobic fermentation can be used to produced hydrogen, the process can be either light dependant or non-light dependant, the latter is called dark fermentation. To produce hydrogen under anaerobic conditions without light, the presence of certain bacteria is necessary (Rodríguez Muñoz, 2015).

(Oliva-Rodríguez et al., 2019) and (Al-Shorgani et al., 2016) reported the isolation of the bacteria of interest by cultivating cultures in special mediums, in anaerobic conditions. (Al-Shorgani et al., 2016) gran stained the cultures and measured the produced gas, only Gram positive and gas producing samples were further studied.

Microorganism are present in nature as mixed populations; it is very rare to find pure cultures. For this reason, specific methods are needed to detect and isolate specific genus and the difficulty to do so varies from genus to genus (Hogg, 2005). The procedures mentioned before to isolate and cultivate the pure cultures are time and cost demanding, therefore, many authors evaluate substrates and inoculums with mixed cultures and depending on the results try pure cultures.

Obergruber, M. *et al.,* (2018) found that in the Czech Republic, the potential demand of hydrogen only in transport is much higher than the country's production capacity. That means that even if the country would use hydrogen as a source of energy for transportation, it would not have enough to supply its needs.

Nonetheless, efforts have been started to be put in place to increase the hydrogen participation as a source of energy. The European Union agreed on a strategy for a smart, sustainable, and inclusive growth of use of hydrogen technologies. In Czech Republic, the Ministry of industry and trade, sponsored the establishment of a hydrogen technology platform, which organizes events and promotes the use of hydrogen. Czechia has accomplished to finish some projects such as a fuel cell bus prototype called Tri-HyBus, a hydrogen filling station, a solid oxide steam electrolyzer, an autarkic system, among others (Obergruber et al., 2018).

It is for these reasons that is important to assess different methods to produce hydrogen, specially the less energy demanding. According to (Rodríguez Muñoz, 2015), dark fermentation is one of the less cost demanding methods to produced hydrogen, because, within the biological methods, as opposed to the photo-fermentation, there is no need of using artificial lighting to ensure that the microorganisms follow the necessary pathways to produce hydrogen; and compared to the most common methods that are heavily energy demanding and use hydrocarbons, dark fermentation is economic and environmentally non-threatening.

Dark fermentation is an important method that should be extensively assessed using the residues from different industries. Evaluating the sludge from breweries in the Czech Republic, is the first step to develop technologies that allow the use of this abundant waste as inoculum/substrate, giving it an added value, as an important energy source and broadening the possibilities to treat wastes in the region.

Authors like (Mizuno et al., 2000), (Ghimire et al., 2015) and (Rodríguez Muñoz, 2015) report disadvantages on using dark fermentation to produced hydrogen, it is mainly done at small scale in laboratories, due to practical issues and low production yields, nonetheless, they highlight the importance of this method to asses residues as potential substrates or inoculums for industrial scale waste treatment.

By doing research on the subject it is possible to encourage the academia to find solutions or alternatives for the practical problems such as low producing yields, hydrogen transportation and storage. Also, this kind of research allows the involvement of the private sector, such as the breweries or companies with other organic residues to use them as potential substrates and inoculums, opening the possibility of private involvement, this brings not only monetary investment, but also skills from people working in the private sector, specific technologies used in those industries, and resources.

4 Literature review

Hydrogen can be found in nature, but normally, it is not mined, therefore it is produced for its different uses. This chapter describes the occurrence of hydrogen in nature, the means of producing it, and its uses.

4.1 Occurrence of hydrogen in nature

Hydrogen is an odourless, tasteless, flammable, and colourless element, at standard conditions is a gas. Nevertheless, at very low temperatures and/or elevated pressures, it can be in liquid or solid state. Normally, it is found as a loose aggregation of two molecules, each of them consisting of a pair of atoms, therefore a diatomic molecule represented as H_2 (Keçebaş et Kayfeci, 2019).

This element is the most abundant in the universe, summing up 75% of the earth's visible matter, stars, and galaxies. It can be found in most of the stars and the sun, and Jupiter is made mostly by hydrogen. On planet earth, the greatest amount is found in water, and very small amounts in the atmosphere (Keçebaş et Kayfeci, 2019). The most common idea about free hydrogen in the atmosphere is that is rare and only present in very small quantities. This preconception has an influence in how much this element is search for, since researchers do not expect to find much free hydrogen, it is not usually sampled, also this affects how detection systems are designed (Zgonnik, 2019).

Despite the common believes, free hydrogen has been found to be present in big quantities in nature, there is documented evidence of wells in East Siberia with daily flows that reach 100,000 cubic meters, or wells in West Africa from which recorded hydrogen extraction dates from 2012 and no decrease of production has been reported (Zgonnik, 2019).The flow of hydrogen in the wells from West Africa is enough to produce the electrical demand for a local village. This is the first case of commercial exploration for naturally produced hydrogen, 18 exploratory wells were drilled in 2018 in search for hydrogen (Prinzhofer et al., 2018).

There are important technical problems in the use of hydrogen, it's transportation and storage, also, it is necessary to develop a new infrastructure for each of those previously mentioned factors (Baird et Cann, 2012). The supply to end-users, including the logistical activities and its storage, is more difficult and expensive for hydrogen than for fossil fuels (Abdin et al., 2020).

Another difficulty with hydrogen extraction compared to fossil fuels is that the drilling for reservoirs is rarely done because its concentrations are mostly in deeper zones, generally in

Precambrian basement, also hydrogen is vastly diffusive and reactive, which diminishes its time contained in geological traps (Zgonnik, 2019).

4.2 Properties of hydrogen

Hydrogen is normally found in three isotopes named 1H, 2H, 3H; 4H and 7H exist but they are very unstable. It has an atomic weight of 1,008 and it has a heat of combustion value of 144000 KJ/Kg (Keçebaş et Kayfeci, 2019). Compared to the conventional fuels, it has the highest energy content per unit of weight, one example is that it has three times more energy content per unit of weight than gasoline (Abdin et al., 2020).

Hydrogen vapor is lighter than air and is combustible in a large range of air/vapor concentration. Hydrogen has an excellent heat conductivity; it can distribute kinetic energy faster than any other gas, this is due to its molecular weight, it is lower than any other gas, because of this, hydrogen molecules move faster and it diffuses faster (Keçebaş et Kayfeci, 2019).

When hydrogen is bonded with other elements, those chemical bonds are form due to share of electrons, most of other elements in the periodic table can form compounds with hydrogen (Keçebaş et Kayfeci, 2019). Those hydrogen containing compounds can be used as energy vectors, they enable the translocation and storage of energy, this can be in the form of a gas, liquid and solid (Abdin et al., 2020).

According to (Menzies, 2019) there are some benefits and some disadvantages of hydrogen as a source or carrier of energy. The beneficial aspect are: It is safe, is not poisonous, it has a very high spontaneous ignition temperature, normally it needs a spark to get ignited; its flammability is not very limiting, its range can go from 3% to 70% H_2 in air mixture, this facilitates a continuous flame; when burnt, it produces water, hence, there are not carbon containing by-products; its flame speed is much higher than methane, 10 times higher.

The downsides are: The higher flame speed rises the temperature of the flame, which consequently produces greater levels of NOx; although stated that safe, its flammability raises concerns of safety; the designs for existing burners have to be reconsidered due to the different Wobbe and CARI indexes in comparison to conventional gaseous fuels such like methane.

4.3 Uses of hydrogen

Hydrogen can be used in industry, transport and in the energy sector. This subchapter describes the different uses of this element.

4.3.1 Industry

Industrial use of hydrogen is widely spread in the industry, in fact, the biggest use of hydrogen is in the manufacture of ammonia, it uses around two thirds of the word's produced hydrogen. (Keçebaş et Kayfeci, 2019). Ammonia is industrially produced mainly under the Haber-Bosch process, which consist on the mixture of nitrogen and hydrogen under temperature and pressure using a metal catalyst (Brown, 2019).

Hydrogen is also used in many hydrodesulfurization and hydrocracking operations in oil refineries. Hydro-desulfurization consist of removing sulphur from refined petroleum products and natural gas; hydrocracking consists in making the molecules of heavy refinery products smaller. In the metallic ore reduction, hydrogen is used to extract tungsten, it can also be used to extract other metals, like copper contained in tenorite and paramelaconite. Hydrogen can also be used in the production of hydrochloric acid when it gets combined with pure chlorine gas in the presence of ultraviolet light. (Brown, 2019).

Food industries also use hydrogen to make unsaturated fats into saturated oils and fats. Atomic hydrogen welding uses hydrogen to weld refractory metals and tungsten. In electrical generators hydrogen is used as a coolant (Brown, 2019).

In various manufacturing plants, hydrogen is used to check for leaks as a replacement of the CCIF3 based gases, which are more environmentally damaging. When producing methane hydrogen is used by mixing it with carbon monoxide in the presence of alumina pellets covered with oxides of zinc and copper, or directly mixed with carbon dioxide. Hospitals and clinics use hydrogen peroxide as a sterilising agent, which is produced when hydrogen reacts with atmospheric oxygen by the means of anthraquinone as a hydrogen carrier. In manufacturing plants of plate gas, hydrogen is used as reducing agent to prevent the formation of stannous oxide. There are many uses of hydrogen for chemical analysis methods, one example is atomic absorption spectroscopy. Gas chromatography uses hydrogen as a carrier phase. Due to hydrogen's low weight, it is used in high altitude weather balloons in meteorological studies (Brown, 2019).

4.3.2 Energy

Hydrogen is considered more as an energy carrier than an energy source, when hydrogen is extracted, the energy used in the process can be stored in the gas, this can be kept, transported and used (Brown, 2019), (Dawood et al., 2020).

Hydrogen can be used as a fuel, and there are two ways of achieving this. In an internal combustion engine, or in a fuel cell. The last one is an energy producing device which coverts chemical energy into electrical by means of an electrochemical reaction of the fed fuel and the oxidizing agent, the cell is basically two electrodes and a membrane placed in between them (Obergruber et al., 2018). Fuel cells operate similarly to batteries, the main difference being that the reactants are supplied continuously. When hydrogen passes through the first electrode, H^+ ions and electrons are produced at the catalytic surface, the electrons go through the external circuit to the second electrode and the ions go through the proton exchange membrane, after that oxygen gas is bubbled producing water (Baird et Cann, 2012).

Hydrogen can also be blended with fossil fuels for conventional internal combustion engines, this has a positive impact in lean-burn capability and flame burning velocity, which reduces carbon dioxide production as a by-product (Chinnici et al., 2018).

4.3.3 Transport

It is possible to use hydrogen in internal combustion engines (ICE) for transport, this kind are very similar to the ones working with conventional fuels, the principal difference is the storage system, is a lot heavier and more complex than the tanks designed for storing diesel or gasoline. Because of that reason, the idea of hydrogen as a fuel for internal combustion engines for on road transportation, is no longer realistic (Abdin et al., 2020).

Internal combustion engines release of some pollutants, it is a common misconception that hydrogen combustion only produces water vapor, but the truth is that it also produces NOx and some hydrogen peroxide. It is important to recognise though, that the amount of NOx produced in internal combustion engine using hydrogen as fuel can be two thirds of the one using diesel or gasoline, and the amount can be eliminated or reduce, using pure oxygen instead of air, or using a catalytic converter (Baird et Cann, 2012).

Fuel cell electric vehicles have an overall efficiency two times higher than internal combustion engine. The vehicles powered by a fuel cell need to carry less hydrogen onboard than the ones using an ICE (Abdin et al., 2020).

Most of the reaction in fuel cells is converted in electricity, but due to the second law of the thermodynamics some of it is converted in heat. Fuel cells have an overall efficiency around 50-55 % much more compared to gasoline using engines with 15-25 % and diesel 30-55% (Baird et Cann, 2012). Because of the reasons above fuel cells are preferred over internal combustion engines.

Fuel cell electric vehicles are pure electrical, they can be used for a long period and can be fuelled in very short time, they can last over 500 km and be charged in a few minutes. Currently storing compressed hydrogen is commercially relevant, used in fuel cell vehicles and refuelling stations. Most of those vehicles use a composite storage system (CSS), to carry the hydrogen in the vehicle, the CSS can be either plastic cylinders or carbon fibre wrapped metal. Where hydrogen is usually stored at 700 bar (Abdin et al., 2020).

Hydrogen can be carried from the production facilities to the retailers in different ways, this depends on the amount of gas needed and the distance of transportation. One option is to transport the hydrogen from a centralised production site using gaseous trucks, the pressure of such trucks is within a range of 35 MPa-50 Mpa, liquified trucks, or pipelines. The second option is that the hydrogen is produced locally at the refuelling stations, this usually use reformers ot small scale electrolysers (Abdin et al., 2020).

There is a great amount of research been done in liquid state hydrogen storage, it has a much higher energy intensity than gaseous hydrogen due to its low volumetric energy density, which is translated in transportation over cost. Hydrogen can also be stored in solid substances, it is a safe and efficient method to store hydrogen for using it in mobile or stationary applications; there are four main groups of solid materials for hydrogen storing: rechargeable hydrides, carbon and other high surface areas materials, thermal chemical hydrides and water reactive chemical hydrides (Abdin et al., 2020).

Various companies in the automotive sector have developed commercial models powered by hydrogen fuel cells, such models are Toyota Mirai, Hyundai Tucson ix35 Fuel Cell or Honda Clarity (Obergruber et al., 2018).

4.4 Hydrogen production

(Obergruber et al., 2018) and (Singh et al., 2015) present different ways to produce hydrogen, either from processing hydrocarbons or by other means including some using renewable sources. By processing hydrocarbons, hydrogen can be produced by gasification of coal, steam reforming of methane, non-catalytic partial oxidation of hydrocarbons and autothermal reforming; or it can be produced by electrolysis, thermal decomposition of sulphate, and by using biomass, hydrogen can be produced by performing some of the same processes carried out with hydrocarbons and still be called biohydrogen, if the compound used it was produced from biomass or using the biomass directly. Figure 4.1 shows the different ways to produce hydrogen from biomass.

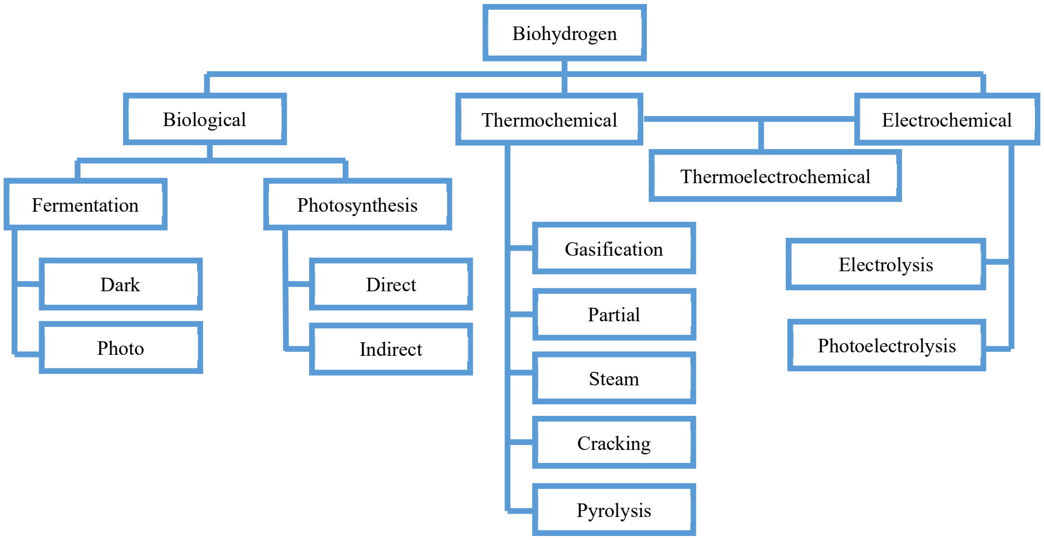

Figure 4.1. Hydrogen production mehtods from biomass. Adapted from (Singh et al., 2015)

From the processes shown before, the biological ones consume the least energy. These biological processes can be divided in fermentative or photosynthetic (Singh et al., 2015), or can also be divided between light and non-light dependant, the light dependant are bio photolysis, photo fermentation, dark fermentation and microbial electrolysis cell (Ghimire et al., 2015).

4.5 Dark fermentation: Process description

Dark fermentation is a process in which complex carbohydrates are transformed into fatty acids, and as a by-product hydrogen, and carbon dioxide are produced (Ren et al., 2009). H_2 is produced when the excess electrons are disposed through the hydrogenase enzyme activity. In anaerobic conditions, protons can performance as electron acceptors to neutralize the electros formed in the oxidation of the substrate, therefore producing the molecular hydrogen H_2 (Ghimire et al., 2015).

The carbohydrates are first transformed into glucose, and then the glucose is converted to pyruvate, through glycolytic pathways, where ATP is produced from ADP and NADH. Pyruvate is oxidized to $CH_3COSCoA$ by the CoA enzyme producing CO2 and H_2 as by products, and depending on the microorganism´s enzymatic systems, the acetyl CoA can be metabolized into Acetate, butyrate, formate or ethanol. In presence of methanogenic bacteria, H_2 is used to produce methane (Ren et al., 2009).

Hydrogen can be produced by a big range of bacteria, but bacteria of the genus *Clostridium* and Enterobacter are the most known to produce hydrogen. The genus Enterobacter are Gram-negative, rod-shaped, and facultative anaerobes and non-spore forming bacteria. While the genus *Clostridium* are Gram-positive, rod-shaped, strict anaerobes and spore forming bacteria (Wang et Wan, 2009). The Table 4.1 shows different species known for high yields of hydrogen production. (Manyuchi et al., 2018) reported that the sludge produced in the process of making opaque beer contains bacteria from the genus *Clostridium*. (Cui et al., 2009) and (Argun et Kargi, 2009) also found that the sludge, lees and wastewater from the beer brewing industry contains hydrogen producing bacteria, they applied pre-treatment methods and obtained good production results.

Table 4.1. Species known for high yields of hydrogen production. Adapted from (Wang et Wan, 2009).

***Clostridium* Species**	**Enterobacter Species**	**Other species**
Clostridium acetobutylicum	*Enterobacter aerogenes HO-39*	*Escherichia coli MC13-4*
Clostridium acetobutylicum ATCC 824	*Enterobacter aerogenes NBRC 13534*	*Escherichia coli*
Clostridium butyricum CGS5	*Enterobacter aerogenes*	*Escherchia coli*
Clostridium butyricum CGS2	*Enterobacter aerogenes HU-101*	*Pseudomonas sp. GZ1*
Clostridium pasteurianum CH4	*Enterobacter aerogenes*	*Thermoanaerobacterium thermosaccharolyticum KU001*
Clostridium paraputrificum M-21	*Enterobacter aerogenes E 82005*	*Thermococcus kodakaraensis KOD1*
Clostridium thermocellum 27405	*Enterobacter cloacae IIT-BT 08*	*Thermotoga elfii*
Clostridium thermolacticum	*Enterobacter cloacae IIT-BT 08*	*Hydrogen-producing bacterial B49*
Clostridium sp. strain no. 2	*Enterobacter cloacae IIT-BT 08*	*Ruminococcus albus*
Clostridium sp. Fanp2		*Hafnia alvei*
		Citrobacter amalonaticus Y19
		Ethanoligenens harbinense YUAN-3

4.5.1 Physicochemical factors

Hydrogen production is determinate by different factors associated with the conditions of the environment. This factors, according to (Show et al., 2011) are, pH, Hydraulic retention time (when hydrogen is being produce intentionally under controlled environments), hydrogen partial pressure, nutrients, temperature, Substrate concentration, microbiological culture and feed stock.

pH - The pH affects the production yield of hydrogen and the metabolic pathways of the bacteria (Ren et al., 2009). According to (Show et al., 2011) and (Show et al., 2011), the maximum yield production of H_2 is obtained in a pH range between 5,2 and 6,0. (Ren et al., 2009) observed that the higher yield production can be obtained at pH around 4,5 in which the ethanol fermentation pathway is the one that occurs the most. (Show et al., 2011) also stated that when the pH is too low or too high, the hydrogenase activity can be inhibited.

Hydraulic retention time - This parameter is most important when hydrogen is being intentionally produced under arranged environments. This factor can be used to select the populations of the microorganisms, if the growth rate of this microorganisms is able to catch up with the mechanical dilution created by volumetric flow. The H_2 producing bacteria have a growth rate of 0,172h-1, and methane producing bacteria have a growth rate between 0,0167 h-1 and 0,02h1. This means that if the hydraulic retention time when adjusted correctly can wash out the methane producing bacteria and retain the H_2 producing ones. When the dilution rate is high the methane production yield is lower. The optimum HRT found in hydrogen producing reactor is of 6h (Show et al., 2011).

Hydrogen partial pressure - The dissolved hydrogen concentration in the liquid phase affects the fermentative hydrogen production. When the hydrogen partial pressure rises, the H_2 production is reduced (Show et al., 2011). The hydrogen partial pressure threshold depends on the substrate and the bacteria species (Steinhauser et Deublein, 2011).

Nutrients - There are many nutrients that are necessary supplements for the nutrition of the microorganisms producing hydrogen, (Show et al., 2011)reported that the most important ones are nitrogen, phosphate, magnesium, sodium, zinc and iron. Nitrogen being preferable in organic compounds because increases H_2 yield production.

Temperature - (Show et al., 2011) say that hydrogen can be produced between 15 and 85. But the higher production is reached under mesophilic conditions, between 30-37 (Yasin et al., 2013).

Substrate concentration - Higher yields of hydrogen production can be obtained from substrate concentration between 10gl-1 and 30gl-1. Although it is debatable if substrate concentrations have a significant effect on the H_2 production because it was found that it is more likely for the microorganisms to suffer starvation from lack iron than from the actual substrate (Show et al., 2011).

The Seed culture - As stated before, there is a big range of hydrogen producing bacteria. On table number 4.1 the higher H_2 producing species are stated (Show et al., 2011).

Feedstock- Simple sugars are easier to degrade in fermentative processes, the gas production period is longer compared to the fermentation of complex sugars. This means that in natural environments where the other factors requirements are met, H_2 production occurs from different feedstock, and only the amount and time of the production variates. In the case of intentional artificial environments, the feedstock is often pre-treated to meet the higher H_2 yield production (Show et al., 2011). The Table 4.2 shows some pre-treatment methods used on the feedstock before applying it to the H_2 producing reactors.

Table 4.2. Pre-treatment methods used on the feedstock. Adapted from (Ghimire et al., 2015).

Pre-treatment methods
Size reduction, <0.297 mm
Size reduction, 1 mm
Hydrothermal (180 C for 15 min)
Steam explosion, 190.220 C for 3–5 mi n
Acidic steam explosion (1.2% H2SO4), 180 and 200 C for 1–3 min
Alkaline at pH 12 using 2 M NaOH for 30 min + microwaves (170 C for 30 min)
100 C for 2 h +4% NaOH (w/v) + cellulase (20 FPU/g)
4% HCl (w/v), boiled 30 min
4% NaOH (w/v), boiled 30 min
1.5% H2SO4, 121 C for 60 min + 9.4 IU/g of cellulase 52 C at pH 4.8 in 0.1 M sodium
citrate buffer at 5% (w/v)
100 30 min and 1% HCl (w/w))

4.5.2 Controlled hydrogen production through dark fermentation

Although hydrogen production from dark fermentation can be achieved in natural environments, studies are made to industrialize this production. Even though the objective is to make assess big scale productions, most studies have been done on laboratory scales. Following, are listed the most important methods and materials to achieve this controlled production.

Dark fermentation in controlled environments is done in a reactor, which can be either continuous or batch type. Most studies of hydrogen production through dark fermentation were done in batch reactors due to their simple operation and control.

Nevertheless, continuous reactors have been utilized and are required for large scales operations, due to practical reasons. In continuous fermentative reactors, Continuous Stirred Tank Reactors (CSTR) and immobilized-cell reactors are widely use. In the first one, biomass is suspended in the mixed liquor which has an equal composition as the effluent. Biomass can be washout at short HRT because the biomass has the same retention time as the HRT. The second one, are an alternative to CSTR, they can retain higher biomass and can operate at shorter HRT (Wang et Wan, 2009).

According to (Boboescu et al., 2014), there are many microorganisms that can produce hydrogen in natural environments, such as soil wastewater sludge, compost, etc. therefore, inoculums can be obtained from this source.

In dark fermentation processes, the use of mixed cultures has proved to have higher H_2 production yields than pure cultures, due to the capacity of mixed cultures to accept a broader source of feedstock. However, if the used mixed culture contains hydrogen consuming bacteria, the H_2 produced will be consumed and the methane will be produced (Rodríguez Muñoz, 2015).

To inhibit the hydrogen consuming bacteria and to prevent their competitive growth, hence affecting hydrogen production yield, there are some reported methods that could be done to different inoculums, such as, Heat-shock, acid, base, aeration, freezing and thawing, chloroform, sodium 2-bromoethanesulfonate or 2 bromoethane sulfonic acid and iodopropane (Boboescu et al., 2014).

The table 4.3 shows some pre-treatments that can be done to different inoculums reported by (Ghimire et al., 2015).

Table 4.3. Pre-treatments that can be done to different inoculums. Adapted from (Ghimire et al., 2015).

Treatment	**Description**
Heat	100 °C for 15 min
Heat	80 °C, 90 °C and 100 °C for 15–30 min
Heat	Heating in boiling water bath for 10–30 min
Heat	105 C for 4 h
Heat	Incubation at 90 C for 1 h
Heat	100–105 C in oven for 2 h
Acid	pH to 2 for 24 h and increasing pH to 5.5 by adding a 2 N NaOH solution
Acid	pH 3 with 2 N HCl for 24 h
Acid	pH to 3 with 1 N HCl for 30 min
Acid	pH 3 with 0.1 N HCl solution for 24 h and adjusting back to pH 7
Base	pH of the sludge to 3 with 1 mol/L of NaOH for 24 h
Base	pH 8, 9 and 10 with 1 mol/L of NaOH for 3 h
Base	pH 12 with 1 M NaOH for 24 h and adjusting back to pH 7 using 1 M HCl
Load shock	Sludge (50 mL) spiked with 40 g of sucrose and acidification for 2 d
Load shock	Sludge (50 mL) spiked with 500 mL of sucrose (50 g/L) and acidification for 2 d
Chemical inhibition	10 mmol of BESA for 30 min and gravity separation for 2 h
Chemical inhibition	0.2 g/L BESA for 24 h
Chemical inhibition	0.1% (v/v) chloroform for 24 h
Aeration	Aerate with air for 24 h
Aeration	Flushing with air for 30 min
Microwave irradiation	Microwave radiation for 1.5 min

After choosing the reactor and the culture, and having pre-treated it, all the factors listed before should meet the best conditions to guarantee the higher H_2 yield production. Table 4.4 shows the parameters influencing the H_2 yield production and the recommended method to insure higher yields.

Table 4.4. Recommended method to insure higher yields.

Parameter	**Recommended method to ensure higher H_2 value**
pH	According to (Ghimire et al., 2015) dark fermentation processes are unstable because of the constant acidity production (VFAs) and since various authors recommend maintaining a pH range between 5-6, the use of buffers to maintain this pH is necessary. However, the use of this buffers is not cost effective and can increase the salt concentration of the effluents, therefore, some authors suggest using substrates with high pH to balance the reactor's pH.
HRT	The optimum HRT found in hydrogen producing reactor is of 6h (Show et al., 2011).
Hydrogen partial pressure	Sparging the reactor with nitrogen (Show et al., 2011)
Nutrients	Add the following nutrients, nitrogen, phosphate, magnesium, sodium, zinc and iron, or find a substrate that contains them (Show et al., 2011)
Temperature	Mesophilic conditions, between 30-37 Celsius are the most recommended. Reactors should be adapted to these conditions.
Substrate concentration	Higher yields of hydrogen production can be obtained from substrate concentration between 10gl-1 and 30gl-1 (Show et al., 2011). This can be stablished when adjusting the weights when setting up the reactor.
Microbiological culture	Please see table 4.3 where inoculum treatments are stated
Feedstock	Please see table 4.2 where feedstock treatments are stated

5 Methodology

The inoculum was obtained from Suchdolský Jeník brewery, which is a commercial, teaching and research brewery part of the life University of Life Sciences, it was founded in 2006 following what the brewery at Brandejs farm in Prague did in previous years (Česká zemědělská univerzita v Praze, 2019).

The beer brewing process is generally carried out in five stages. The first one is the malting where barley is germinated; the second is the mashing in which the extraction and hydrolysis of the malt take place and non-soluble components are separated, in simpler terms, the wort is separated from the sludge; the third stage consists in the boiling of the wort, and is in this phase where the hops are added; in the next stage the fermentation takes place; and finally the fifth stage is called down-stream processing, where the filtration, stabilization, pasteurization and bottling happen (Basanta et al., 2008).

The inoculum was taken from the sludge at the second stage of the brewing process. In Suchdolský Jeník brewery the separation of the sludge and wort takes place in a special vessel called whirlpool, due to the high speed swirl in this vessel, the sludge forms a cone in the centre as a result of the Einstein effect (Česká zemědělská univerzita v Praze, 2019). In Figure 5.1 is possible to see the whirlpool and the sludge that was used.

Figure 5.1. Suchdolský Jeník brewery's whirpool with sample sludge.

Later the inoculum was subdued to pre-treatments processes to enhance the productivity, the two pre-treatments were pH adjustment with a buffer and heat shock. The pH adjustment was done before and after the heat shock.

Sodium bicarbonate has a very high solubility, and when dissolved generates great concentrations of bicarbonate ions and pH increases (Sampathkumar et Gothandam, 2019). $NaHCO_3$ as a buffer has shown to have a positive impact in anaerobic digestion processes, it increases biodegradability of organic waste and biogas production (Gao et al., 2015).The following is the procedure to determinate the amount of buffer to adjust the pH to the preferred value. Based on the literature, a value of 5,65 was chosen as optimal pH, and the chosen buffer to maintain this value was NaHCO3.

The paper pH meter was considered but due to its inaccuracy and the nature of the material, this option was discharged and the probe pH meter was chosen for the measurement of the pH with the probe pH meter, the chosen procedure was the one suggested by the Environmental Protection agency (EPA) in the METHOD 9045D, which is used for soils and waste, this waste can be solid, sludges and non-aqueous liquids (Environmental Protection Agency, 2004).

Following the method 9045D, the pH meter was calibrated at minimum of two points, a sample of the sludge was taken and was dissolved with distilled water, this solution was centrifugated to properly separate the solid particles and to have a totally liquid solution, which facilitated the measurement with of the pH with the pH meter. The pH value found in the sample was 2,97. See figure 5.2.

Figure 5.2. Initial pH measuring and value of the sludge

Subsequentially 50g of sludge were taken as a sample to determine the amount of necessary buffer, this 50g were mixed with distilled water to have a solution in which the pH could be measured. With a pipet a 5% solution of $NaHCO_3$ was slowly added to the sludge solution, the solution had a magnetic mixer which assured the homogeneity of this one, a pH meter was measuring the pH the whole time.

The 5% solution of $NaHCO_3$ used to find the optimal pH value was prepared with 100ml of distilled water and 0,5g of $NaHCO_3$. The needed amount of the solution was found to be 55ml for 50g of wet sludge, using the following calculation shown in Equation 5.1, the necessary volume of buffer for 300g of wet sludge was found.

Equation 5.1. Calculation of the buffer's volume.

$$Volume\ of\ NaHCO3\ (5\%) = \frac{\text{55 ml NaHCO3 Solution x 300 g sludge}}{\text{50 g sludge}} = 330\ ml\ \text{ NaHCO3 Solution}$$

The total mass of sodium bicarbonate was found to be 1.65 g, this value corresponds to the 5% concentration and the desired volume of 330 ml, and it was calculated by means of the Equation 5.2. After having the total buffer solution, the pH was measured once again, to assure that the optimal value was obtained, and it was achieved as expected.

Equation 5.2. Calculation of the mass of buffer to be used.

$$grams\ of\ NaHCO3 = \frac{\text{330 ml Solution x 0,5 g NaHCO3}}{\text{100 ml Solution}} = \text{1,65 g NaHCO3}$$

When non isolated inoculums are used, such as waste sludges, adjusting the environments to favour hydrogen producing bacteria (HPB) is essential, for this reason the pH was controlled with a buffer and the sludge was subject to a heat treatment. According to (Hogg, 2005), sterility is normally reached by boiling at 100 °C by 10 minutes, and most bacteria are killed at about 70 °C; but endospores can resist boiling even for prolonged time e.g. hours, it is required to autoclave at 121°C; the spores can resist because of their thick coat that surrounds them. The genus *Clostridium* are Gram-positive, rod-shaped, strict anaerobes and spore forming bacteria (Wang et Wan, 2009). In order to favour HPB such as *Clostridium* and to eliminate others, the inoculum was put into the oven to go through the second pre-treatment method, a heat shock. This was done in the laboratory's autoclave; the temperature was 90°c and the time was set for 1 hour. After the heat sock the inoculum was left aside for one day, to allow the spores to germinate into new bacterial cells. In figure 5.3 it is shown the inoculum after this process. From the whole amount of dry sludge, 25 grams were separated for each reactor.

Figure 5.3. Inoculum after heat shock.

The pH was measured again and the found value was 4,67. By using the same procedure as before but with a sample of 3g of sludge, it was found that 6 ml of the buffer solution were needed and the pH was adjust again to 5,65. See Figure 5.4.

Figure 5.4. Last pH adjusted measurement.

To ensure, that the substrate was not a restrictive factor, it was decided to add glucose as feedstock, the amount of glucose was determined according to the methodology of (Mizuno et al., 2000), for the total volume of each reactor 6 grams of glucose were used. Carbohydrates such like glucose are the ideal source for fermentation processes, their presence increases the production of butyric acid and hydrogen gas (Mizuno et al., 2000).

After all the required volumes and masses were determined, everything was mixed in three identical bottles, and filled until the desired total volume of 600 ml. To close the reactors, the lids were perforated with a syringe needle in order to connect a hose to each bottle, the needle shafts were properly tightened, but to ensure that no gas would escape, parafilm was used in the surroundings of the needle shaft inserted in the lids. Parafilm is a paraffin wax tape, it is used to seal laboratory equipment and can resist a big range of temperatures, from -45 °C to +50 °C (Bemis Company Inc., 2005)(Heathrow Scientific, 2012). That means that the reactor conditions will not affect the sealing.

Each reactor was marked in order to record separated results. Figure 5.5 shows the three reactors filled, closed, and connected to the needles that serve as a tube to transport the gas.

Figure 5.5. Reactors ready to be put in constant mesophilic conditions.

HPB are known to be able to produce the gas in ambient temperature, but it has been observed that increasing the temperature to mesophilic conditions always improve the hydrogen production, specifically at 35 ± 1 °C (Show et al., 2011). Because of that reason the three bottles were placed in a heated circulating bath at a set temperature of 35 °C. The heat conducting material was distilled water, municipal tap water should not be used unless it meets the minimum requirements of the operators' manual after tested, the main reason being the corrosion caused by the dissolved solids and or a non-neutral pH value (Pratt, 2016).

Each hose was connected to the reactors using the needle hub, the diameter of both parts permitted that there was a good sealing, preventing the produced gas to scape.

According to (Chang, 2010), a gas that it is being bubbled through a hose can be collected over water if it that has low solubility and reaction with the water, this method is called water displacement and Figure 5.6 shows a schematic example of oxygen being collected with this method.

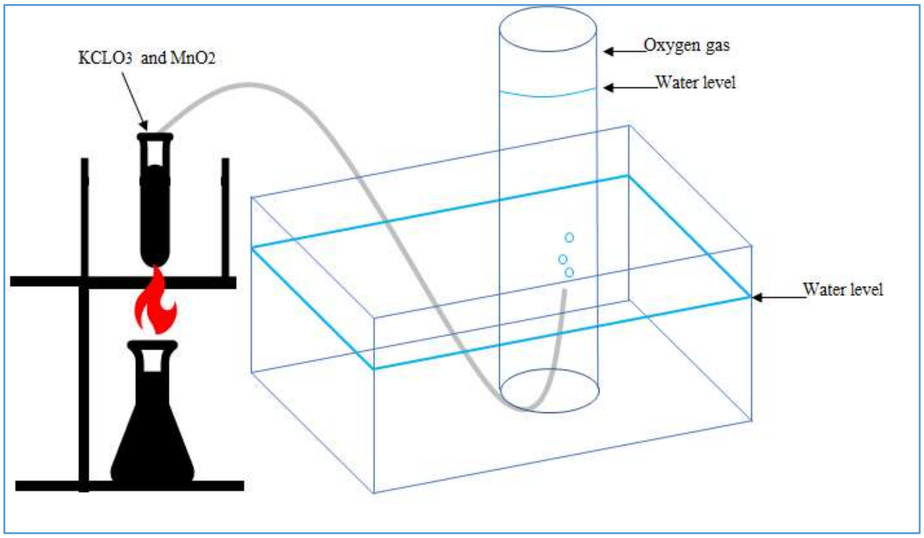

Figure 5.6. Scheme of Oxygen gas collected by water displacement. Adapted from (Chang, 2010).

According to (Dawood et al., 2020) hydrogen meets the solubility and reactivity conditioned by (Chang, 2010), hence the water displacement method was chosen to collect the gas. Each hose was introduced in an inverted measuring cylinder, which were filled with water and submerged in another recipient with water, this way the gas can push the water away and this volume could be measured. See figure 5.7.

Figure 5.7. Reactors placed in optimal conditions and connected to the gas collecting system.

6 Results

After setting up the reactors, the volume of the produced gas was monitored until it got stabilized, this was after 1 and 2 days for reactors 2 and 1 respectively, the reactor number three did not show any gas production.

The gas produced displaced the water in the inverted measuring cylinders, and the volume of gas was measured. The Figure 6.1 shows the gas and the displaced water in the collecting cylinders for each reactor connected by the hose.

In the collector on the front, corresponding to the third reactor there is no gas and it is completely filled with water. Oppositely, the cylinder in the middle, which corresponds to the reactor 3 shows some volume of water displaced.

Finally, the one in the back, shows the biggest volume of water displaced and thus the maximum gas production.

Figure 6.1. Inverted measuring cylinders with the collected gas after the production was stabilized

The reactor 2 started showing signs of gas production 5 minutes after placed in the heated circulating bath, it was also the first to stabilize after 1 day, followed by the second reactor on the next day with a lower gas volume. The results of gas produced by each reactor are shown in Table 6.1 in millilitres.

Table 6.1. Produced gas results in millilitres.

Reactor 1	60 ml
Reactor 2	145 ml
Reactor 3	0 ml

(Ghimire et al., 2015) reported that when hydrogen is being produced the maximum theoretical value of H_2 is 4 mol for each mole of glucose, this balance can be used to calculate the theoretical H_2 production in a controlled reactor where the substrate is carefully measured before used. See Equation 6.1.

Equation 6.1. Acetic acid forming metabolic pathway from glucose. Adapted from (Ghimire et al., 2015).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CO_2 + 4H_2 + 2CH_3COOH$$

In the Equation 6.1 the final product is acetic acid instead of acetate as describe in the metabolic pathway mentioned before. The explanation behind this is that when the formula of acetic acid is expressed as CH3COOH, it is an indication that the ionizable proton is in the group COOH, acetic acid is a weak acid and gets ionized into acetate, this reaction occurs in both directions, acetic acid breaks into acetate and hydrogen ions and sometimes these last two recombine to form acetic acid again (Chang, 2010). See Equation 6.2.

Equation 6.2. Ionization of acetic acid. Adapted from (Chang, 2010).

$$CH3COOH(aq) \leftrightarrow CH3COO^{-}(aq) + H^{+}(aq)$$

When the maximum hydrogen production (MHPY) yield is achieved, the metabolic pathway follows the next steps, first the glucose is converted to pyruvate, though glycolytic pathways, where ATP is produced from ADP and NADH, subsequently pyruvate is oxidized to CH3COSCoA by the CoA enzyme producing CO_2 and H_2 as by products, the microorganism's enzymatic systems metabolize the acetyl CoA into Acetate (Ren et al., 2009). In Figure 6.2 there is an schematic description of the metabolic pathway previously described.

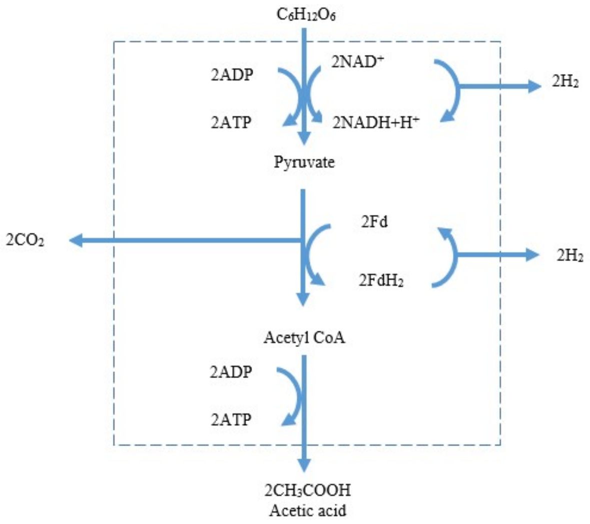

Figure 6.2. Metabolic pathway from glucose to acetic acid and sub products. Adapted from (Ren et al., 2009).

Based in Equation 6.3, the maximum theoretical yield of hydrogen was found to be 0,13 mol, the procedure is described in the following calculation, Equation 6.3.

Equation 6.3. Calculation of theoretical hydrogen moles.

$$6\ g\ C_6H_{12}O_6 \times \frac{1\ mol\ C_6H_{12}O_6}{180\ g\ C_6H_{12}O_6} \times \frac{4\ mol\ H_2}{1\ mol\ C_6H_{12}O_6} = 0{,}13\ mol\ H_2$$

The pressure of the gas had to be calculated in order to find the moles of the produced hydrogen for each reactor, this using the water displacement technique and the ideal gas equation. For all calculations, the following parameters were used: room temperature of 25°C and pressure of 975hPa, this last value was taken from the meteorological station of Czech University of life Sciences (Meteorologická stanice České zemědělské univerzity v Praze, 2019), at the time and date the gas volume was last measured, 21.11.20129 15:00.

According to (Chang, 2010), Dalton's law can be used to calculate the pressure of the produced gas. The total pressure is equal to the sum of the produced gas and the water vapor. According to this, the Equation 6.4 was deduced, taking the atmospheric pressure as total pressure because the gas was collected over water at atmospheric pressure.

Equation 6.4. Dalton's law of partial pressure applied. Adapted from (Chang, 2010).

$$P_g = Patm - P_{H2O}$$

P_g= Pressure of the produced gas.
Patm= Atmospheric pressure.
P_{H2O}= Pressure of the water vapor. $^{Note.\ 1.}$

Note. 1. Water vapor pressure at 25°. 23,76 mmHg (Chang, 2010).

P_g=97500 Pa - 3167,739474 Pa
P_g= 94332,26053 Pa

To compare the results to the theoretical yield, first the pressure of the hydrogen molecules had to be calculated. Being hydrogen 66,66% and carbon dioxide 33,33% of the gas, the pressure of the produced gas was multiplied by the 66,66% to find the hydrogen partial pressure. As a result, it was found that the partial pressure of the hydrogen is 62891,3181 Pa.
This calculation was based in the Equation 6.1 and Dalton's law of partial pressure. This can be achieved by the means of mole fraction (Chang, 2010). See Equations 6.5 and 6.6.

Equation 6.5. Molar fraction equation. Adapted from (Chang, 2010).

$$X_i = \frac{n_{\mathrm{H2}}}{n_T}$$

X_i= Mole fraction.
n_{H2}= Moles of hydrogen.
n_T= Total moles of the gas.

X_i= 4 mol H_2 / (4 mol H_2+ 2 mol CO_2)
X_i= 4 mol H_2 / 6 mol (H2+CO2)
X_i= 0,6667

Equation 6.6. Partial pressure of hydrogen equation. Adapted from (Chang, 2010).

$$P_i = X_i P_T$$

P_i= Partial pressure.
X_i= Mole fraction.
P_T= Total pressure.

P_i= 0,6667 x 94332,26053 Pa

P_i= 62891,3181 Pa

To find the experimental moles of hydrogen, the ideal gas equation PV=nRT was used, shown in Equation 6.7.

Equation 6.7. Ideal gas equation. Adapted from (Chang, 2010).

$$n = PV/RT$$

n= moles of the produced gas.
P= Pressure of the produced gas.
V= Volume of the produced gas.
R= Ideal gas constant.
T= Temperature of the gas.

Reactor 1.

$n = (62891,3181 \text{ Pa} \times 6 \times 10^{-5} \text{ m}^3) / (8,3145 \text{ JK}^{-1}\text{mol}^{-1} \times 298 \text{ K})$

$n = 1,523 \times 10^{-3}$ mol

Reactor 2.

$n = (62891,3181 \text{ Pa} \times 1,45 \times 10^{-4})/(8,3145 \text{ JK}^{-1}\text{mol}^{-1} \times 298 \text{ K})$

$n = 3,680 \times 10^{-3}$ mol

Reactor 3.

n = 0 mol

The results show that the moles of hydrogen generated by the reactors is significantly lower than the theoretical value. The theoretical value is 0.13 moles of hydrogen, 35 times higher than the yield of reactor 2, which had the maximum hydrogen production, and 86 times higher production than reactor 1, reactor 3 did not have any production. Please see Figure 6.3.

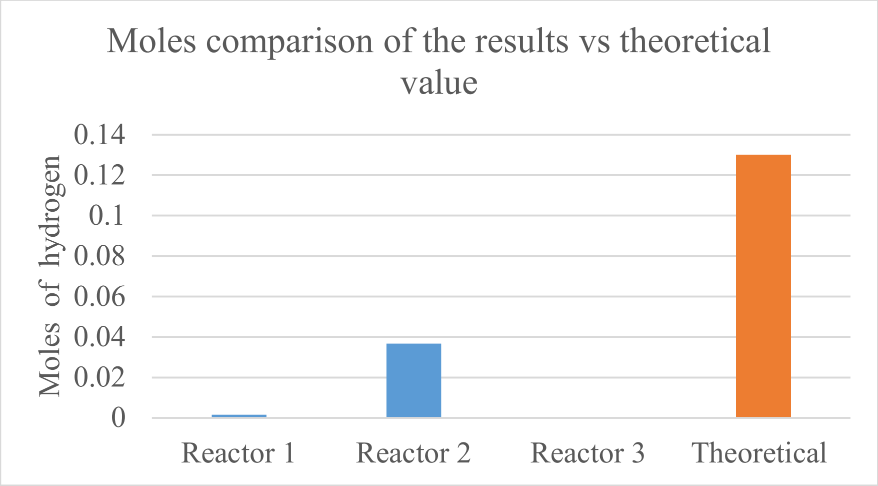

Figure 6.3. Comparison of results and theoretical yield in moles

To compare the difference between the results and the theoretical yield, the percentage yield was found, and named efficiency (η) for practicality. According to (Chang, 2010), the percentage yield is the ratio of the empirical yield to the actual one, multiplied by 100 to express it in percentage; the theoretical yield of a reaction is determined by the amount of limiting reacting, and it is the maximum yield obtained based on the balanced equation, the author also states that the percentage yield is normally less than 100%. Equation 6.8 was used to calculate the percentage yield expressed as ɳ.

Equation 6.8. Efficiency comparing experimental yields with the theoretical yield. Adapted from (Chang, 2010).

$$\eta = \frac{mol_E}{mol_T} x\ 100$$

ɳ = Efficiency
mol_E= Experimental moles.
mol_T= Theoretical moles.

Reactor 1.

ɳ= (1,523 x10^{-3}mol / (0,13mol) x 100

ɳ= 1.17%

Reactor 2.

ɳ= (3,680 x10^{-3}mol / 0,13mol) x 100

ɳ= 2.83%

Reactor 3.

ɳ= 0 %

The results show that very little of the theoretical yield was achieved, only 1.17% for the Reactor 1, 2.83% for the Reactor 2 and 0% for the Reactor 3. Figure 6.4 shows a schematic representation of how much of the theoretical yield was achieved in each reactor.

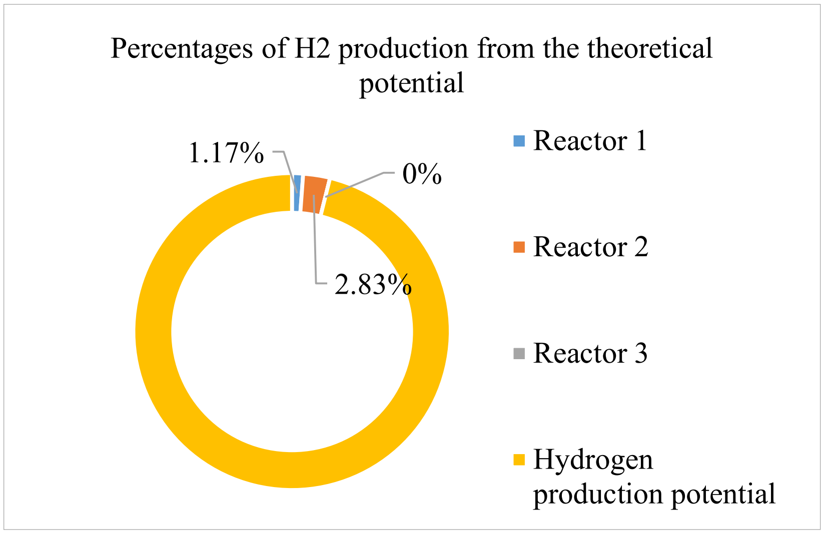

Figure 6.4. Percentage yield comparison to theoretical yield.

To summarize, table 6.2 shows the results obtained as gas volume and based on those, the calculated hydrogen moles, and percentage yield.

Table 6.2. Results summary.

	Gas produced	**H_2 moles**	**Efficiency**
Reactor 1	60 ml	1,523 x10^{-3} mol	1.17%
Reactor 2	145 ml	3,680 x10^{-3} mol	2.83%
Reactor 3	0 ml	0 mol	0 %

7 Discussion

The efficiency was calculated based only on the amount of extra glucose that was added. The amount of glucose in the sludge is very limited and it may not be completely bioavailable, therefore, is neglected. In typical brewing worts around 90% of the carbohydrates from the cereal are present, which contain sucrose, fructose, glucose, maltose and maltotriose and dextrins, from which glucose is only 20% (He et al., 2014).

The sludge used, also called in literature brewery spent grain is composed principally of barley husks, the seedling, part of the pericarp and other materials that were not solubilized in the mashing process. Only the soluble substances from the grain become part of the wort, they become part of it by the action of enzymes that degrade the starch into sugars and soluble dextrins, and proteins and lipids degrade to low molecular weight products. On the other hand, insoluble substances, such as non-solubilized or nondegraded starch, a portion of the high molecular weight protein, cellulose and other compounds remain in the sludge (Skendi et al., 2018).

(USDA, 2019) state that each 100g of dry barley contains about 72,73g of carbohydrates (COH). According to the percentages of (He et al., 2014), 14.546 g of 100g grams of dry barley would be glucose, from which 1.4546 g would be left in the sludge after the wort extraction, leaving finally 0.36365g of glucose in the 25g of dry sludge placed in each reactor. See in Equation 7.1.

Equation 7.1. Calculation of glucose in dry sludge.

$$glucose\ \ in\ sludge\ (g) = \frac{\text{25 g Sludge x 1.4546 g glucose in 100g barley}}{\text{100 g of barley}} = \text{0.36365 g}$$

In terms of bioavailability, (Geng et al., 2010) reported that there are certain kind of bacteria that can produce hydrogen from cellulose fermentation, those kind of bacteria are called cellulolytic, and include one strain of *Clostridium* called *Clostridium thermocellum,* nonetheless, their use is limited due to the pH sensitivity, poor growth rates and low hydrogen yields. (Morales-Martínez et al., 2020) added to their reactors a microbial consortium from ruminal origin, which help to release sugars from cellulose and make them available to *Clostridia* to degrade them, they state that in general the genus *Clostridium* does not produce enough cellulolytic enzymes to degrade by themselves cellulose. Since the pre-treatments done to the brewery sludge were specific to enhance the growth of *Clostridia*, and the most of the components of the sludge are not bioavailable to the bacteria of interest, the content of glucose present on the sludge was neglected.

According to (Heathrow Scientific, 2012), long exposure to polar substances can cause embrittlement to the parafilm, considering that acetic acid is a polar substance and during the fermentation the liquid part was in contact with the lids, small fissures could enable some gas to scape therefore contributing to the low efficiency of the reactors since it was calculated based on the volume of the gas. However, the low content of gas on the measuring cylinders is more likely to be caused by the absence or little presence of hydrogen producing bacteria.

The sludge presented an initial pH lower than 5, after applying the $NaHCO_3$ as buffer, the ideal value was achieved; however, after the heat shock pre-treatment was seen to be slightly reduced and it was readjusted again to 5,65. The initial conditions of the reactor were ideal, and an eventual reduction of the pH was expected due to the formation of the acetic acid; nonetheless it is anticipated to be slowed and minimized by the application of the buffer (Meky et al., 2020). Nevertheless, an abrupt pH drop could have caused the inhibition of the HPB in all reactor, especially Reactor 3. According to (Meky et al., 2020), when the pH is lower than 5, microbial activity, including hydrogen producing bacteria, is inhibited.

(Meky et al., 2020), also suggest that for hydrogen production when the substrate is mainly composed by gelatine, and the reactor is at 30°C, the optimal pH value is 6,3. The operational conditions of the Reactors 1,2 and 3 were different based on the literature, nevertheless, the presence of gelatine in the sludge was not considered. If part of the sludge was gelatinized, the chosen pH of 5,5 might have not been ideal.

According to (Skendi et al., 2018), the drying time and high temperatures do not have a major effect in the nutrient content of the sludge, but it influences in the decrease of the undigested starch content, which is related to the partial gelatinization of starch. Since the sludge was pre-treated with a heat shock, it might have occurred a partial gelatinization of the remaining starch, making the pH proposed by (Meky et al., 2020) the ideal, as opposed to the one used in the reactors, contributing to the low hydrogen yield production.

(Ghimire et al., 2015) state that due to the metabolic nature of dark fermentation, the pH of the reactors is prone to become acidic because of the formation of organic acids. The authors made an extensive literature comparison of the ideal pH value, finding the best yields between 5 and 6, and suggesting maintaining those values using buffers, or slightly basic substrates, reducing costs in buffers.

As opposed to (Ghimire et al., 2015), other authors suggest to set the reactors at a higher initial pH, using bigger amounts of buffer, this could inhibit the growth of the bacteria of interest at the beginning, but once is lower, the endospores would germinate and the hydrogen production yield would increase.

(Mizuno et al., 2000), (Geng et al., 2010), (Meky et al., 2020) and (Kumar et al., 1995) reported their MHPY using an initial pH between 6.5 and 7, the comparison of their maximum yields can be observed in the Table 7.1, where (Meky et al., 2020) reported a MHPY of 0.4 litres per grams of initial COD using an initial pH of 6.5; both (Mizuno et al., 2000) and (Geng et al., 2010) reported a maximum yield with a pH of 6.8 with values of 2.52 and 1.36 mole per mole of glucose respectively; (Kumar et al., 1995) found a maximum yield of 34 litters per mole of glucose with an initial pH of 7.

To be able to compare the maximum production yields of the other authors with the results from the three reactors used in this trial, the maximum yield of the reactor number 2, which was the one that produced the more gas, was converted to the different units in which the other authors expressed their yields. To be able to compare the data with (Meky et al., 2020) in relation to COD, the theoretical average COD value of 1400 g/kg proposed by (dos Santos Mathias et al., 2015) and (Vitanza et al., 2016) was used. See Table 7.1.

Table 7.1. Comparison of MHPY in relation with the initial pH

Max. Yield		**Units**	**Initial pH**		
Current study	**Comparison**		**Current study**	**Comparison**	**Reference**
$3.54x10^{-3}$	0.4	L H2/ g COD	5.65	6.5	**(Meky et al., 2020)**
0.11	2.52	Mole H2/ mole Glucose	5.65	6.8	**(Mizuno et al., 2000)**
0.11	1.36	Mole H2/ mole Glucose	5.65	6.8	**(Geng et al., 2010)**
4.35	34	L H2/ mole Glucose	5.65	7	**(Kumar et al., 1995)**

(Rodríguez Muñoz, 2015) reported an increase of almost 11% in the hydrogen production yield in the dark fermentation trials by increasing the initial pH from 5.5 to 7, the author also increased the total yield by subsequently fermenting the organic acids produced during the dark fermentation, using light as a catalyst.

The growth and even viability of most bacteria is normally negatively affected by the presence of organic and inorganic acids, depleting the activity of biological macromolecules. Normally bacteria are able to maintain a fairly stable internal pH, they possess a transcriptional and translational response to a pH reduction; if this response is reduced or prevented, the bacteria can be endangered, when acid stress is too strong, the internal pH can be reduced to levels that are not possible to correct by buffering, ionic flux or inducible responses. Organic acids have such negative impact on bacteria at pH values lower than 5 because they transport molecules into the cytoplasm in the hydrophobic unionised form and subsequently dissociate, reducing the intracellular pH (Lund et al., 2014).

According to (Jongenburger et al., 2015), microorganisms are presumed to be homogeneously distributed through a batch that has been treated under the same conditions; however, the authors propose 5 scenarios in which bacteria can be distributed in a non-homogenous manner and presenting different levels of presence. See Figure 7.1.

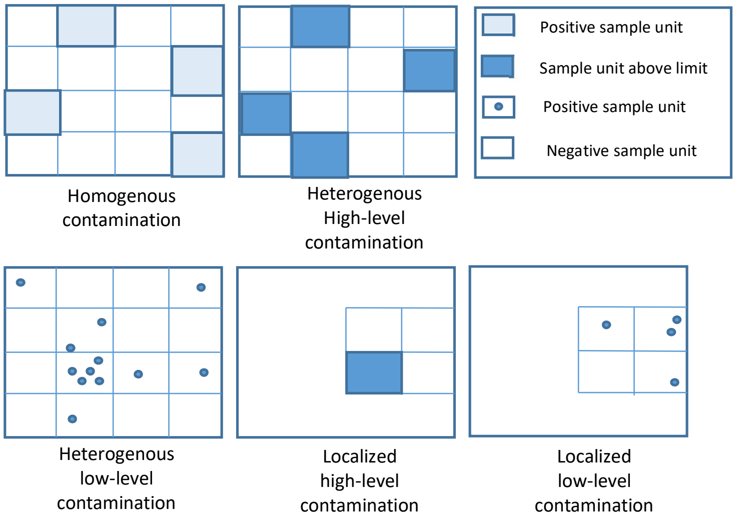

Figure 7.1. Scenarios of microbial distribution proposed by (Jongenburger et al., 2015) . Adapted from (Jongenburger et al., 2015)

The absence of gas production in Reactor 3 compared to the other two could be explained partly by the distribution of the microorganisms. The sludge sample taken from the brewery was treated as one sample divided in three batches, the pH and heat pre-treatments were used to ensure that only spore forming and HPB could survive, the whole microbial community was reduced, thus the bacterial distribution was depending solely on the endospores, if the sample of the sludge that was used presented a distribution like the last two scenarios, where the presence of the bacteria or in this case endospores were concentrated in only a few spots, the Reactor 3 might not have any presence and thus not produced any gas. Or it could have had very little concentrations of endospores and the not favourable conditions, like the abrupt pH drop, limited the possibility of the new cells to activate. For this reason, some authors suggest after assessing certain products as a potential inoculum, with good results, to isolate, cultivate and make trials with pure cultures.

(Al-Shorgani et al., 2016) isolated a strain of *Clostridium* from a soil in Malaysia, they Gran stained the cultures and cultivated in a special medium and the produced gas was measured and only Gram positive and gas producing cultures were further studied.

(Oliva-Rodríguez et al., 2019) reported that to propagate *Clostridium* strains such as *Clostridium acetobutylicum ATCC 824*, *Clostridium beijerinckii ATCC PTA 1550* and *Clostridium beijerinckii NCIMB-8052*, they needed to cultivate the cultures in a synthetic medium in bottles at a specific volume, a pH of 6.7 using glucose and the following nutrients and buffers, MgSO4·7H2O, MnSO4·H2O, KH2PO4, K2HPO4·3H2O, FeSO4·7H2O NaCl, ammonium acetate, p-aminobenzoic acid, biotin, thiamine, yeast extract, cysteine and resazurin solution, under strict anaerobic conditions ensured by nitrogen purging and tight sealing.

(Puskarčík, 2013) state that beer production in Suchdolský Jeník brewery, involves boiling the grain several times, this could have a negative effect in the hydrogen producing bacteria, and even in the survival of the spores, affecting later the hydrogen yield.

According to (Rodríguez Muñoz, 2015), the MHPY can be influenced by the time the inoculum has been cultivated, the freshness of the inoculum might change the relative abundance of the microbial diversity in mixed cultures.

The inoculums in the three tested reactors were composed of mixed cultures, and the incubation time and the delay between that time and the fermentation might have had an effect on the microbial diversity in a negative way towards *Clostridia*.

As mentioned in chapter 4.5.2, there are several methods to adjust the inoculum and substrate to the ideal conditions to facilitate the growth of the bacteria of interest.

(Alibardi et al., 2012) reported a case study where for sludge two pre-treatment methods were tested, different values of pH and time exposure to heat were compared, they tested the hydrogen production at pH values of 5.5, 7.0, and 8.5 during 0.5, 1, 2 and 3 hours of heat exposure. They found that the hydrogen yield found at a 5.5 pH for 4 hours is almost twice higher than the yield found at the same pH for 1 hour.

The last pre-treatment conditions in which they found the lowest yield, are similar to the ones used in this study, which indicates that a longer exposure time to heat could have been a more suitable pre-treatment to the inoculum, increasing the MHPY.

(Alibardi et al., 2012) also suggest that no methane was detected in their results at any of the tested conditions, ruling out the presence of methanogenic bacteria; but they found that by reducing the heat exposure time from 4 hours to 1 hour, some other genus of bacteria could have survived due to the physical characteristics of the sludge, affecting negatively the hydrogen yield, due to the competition of those other bacteria with the hydrogen producing ones.

Iron can be a limiting factor in hydrogen production in dark fermentation, it is a key component in the enzymatic activity of the microorganisms involved in the implicated metabolic pathways (Show et al., 2011).

(Wang et Wan, 2009) state that the metal ion is indispensable for the hydrogenase enzyme, which has a big role in the metabolic formation of fermentative hydrogen, but they also state that an elevated concentration of metal ions can inhibit the hydrogen producing bacteria. Microorganisms use two unrelated enzymes to either produce or to oxidize molecular hydrogen, those two enzymes are Ni-Fe hydrogenase and Fe-only hydrogenase; the later one is limited to strict anaerobes and it is highly sensitive to molecular oxygen (Pandey et al., 2008).

(Dhar et al., 2012) report that iron-sulphur clusters are necessary cofactors of proteins, they state that a wide range of iron concentration have been studied, between 0 and 14000 mg/L, additionally it is mentioned that adding iron also helps to control sulphide concentrations in anaerobic conditions; nevertheless, the precipitation of sulphide as ferrous sulphide has a negative impact in the hydrogen production, because the iron is not bioavailable for the microorganisms.

Extra iron was not added to any of the reactors. Considering the natural content of iron in barley, which is, according to (USDA, 2019) 3,27 mg per 100g of barley, each reactor is ought to have 0,8175 mg according to the dry mass of barley used for each bottle, that translates in a concentration of 1.3625 mg/L.

Iron in food occurs in three main forms, Fe^{2+} ferrous iron, Fe^{3+} ferric iron and ferrous iron chelated into a complex organic compound; the first two are associated to iron from plants (non heme iron) and the last one to the animal iron (heme iron) (Scientists, 2013). (Wang et Wan, 2009) state that the most important form of iron is Fe^{2+} due to its importance in the metabolic path forming hydrogen. Non heme iron requires an acidic pH to get reduced becoming Fe^{2+} from Fe^{3+} when in those conditions.

The conditions in the reactor therefore ensure the bioavailability of the iron in the ferrous form for the hydrogen producing bacteria. However, the low concentration in the barley could have been a limiting factor in the hydrogen production.

The literature is not consistent in the optimal amount of iron needed to optimize the H_2 production yield, nonetheless, the lowest value reported as optimal by (Wang et Wan, 2009) is 10 mg/L, considering that the iron concentration for each reactor was 1.3625 mg/L, assuming that all iron became bioavailable, it is possible that this metal ion was a restricting factor for the H_2 producing bacteria, and the yield was compromised.

The order of the bottles in relation to the heating unit was changed after noticing that the bottle that was put first next to the heating unit (Reactor 2) was the first one to start producing gas. According to (Raedler et al., 2016), there are five factors that influence in the performance of the thermal bath circulator, which are, stability, accuracy, annual drift, digital setting accuracy and uniformity; they should be calibrated to assure that any object that is being kept at a set temperature in the system, really is.

The circulating heat bath was tested before and proofed to be working fine, but the last meticulous calibration was done months before. There can be a relation between the quick start of gas producing and the proximity to the heating unit; (Raedler et al., 2016) state that the uniformity in circulating heat baths, refers to the homogeneity of the water temperature in the system, and it can vary from the control sensor temperature at different locations and depths. The Reactor 2 not only showed to be the first reactor to produced gas, but also the one having the highest yield, if the uniformity of the system was not at its optimal setting, this result could be partly attributed to the initial proximity to the heating unit, giving an initial better condition to the endospores to activate compared to the other reactors.

(Raedler et al., 2016) also stated that the stability can be affected by the selected setpoint temperature, the fluid used as a heat transfer, the method of controlling the temperature, the heating and cooling power, the length of the pipes and the pumping performance.

Even though the screen showed 35 °C during the entire time of the fermentation, stability could have been affected by any of the factors listed above, 35 °C was used as the ideal temperature based on the findings of (Show et al., 2011), a variation of that temperature could have had an influence in the low yield of the reactors.

When a biological reaction takes place, normally, it must be exergonic, meaning that the free energy should be negative. In the case of hydrogen/methane formation, hydrogen has to be well balanced, when there are methanogenic bacteria, there should be enough hydrogen for these bacteria to form methane, but not too much so the acetogenic bacteria are not surrounded by an excess and stop producing hydrogen. The hydrogen partial threshold varies depending on the species of bacteria and the nature of the substrate (Steinhauser et Deublein, 2011).

There are methods such as sparing the reactors with N_2 and CO_2, applying a H_2- permeable membrane and applying a vacuum pump to reduce the partial pressure, but those are used in CSTR reactors (Ghimire et al., 2015). The reactors used this trial were batch type, thus these methods to reduce the hydrogen partial pressure, could have not been applied. Nonetheless, an elevated H_2 partial pressure could have diminished the production of the gas of interest.

(Rodríguez Muñoz, 2015) suggest that by combining the photo-fermentation and dark fermentation there is an increase of the H_2 production yield, by using the organic acids produced during the dark fermentation as substrates for the photo-fermentation.

By using photo fermentation there is a theoretical increase of 4 moles of hydrogen, per the initial mole of glucose used during the dark fermentation if it is thee degradation of acetic acid (Rodríguez Muñoz, 2015).

The Equation 7.2 shows the general equation of hydrogen production per mole of organic acid, having carbon dioxide also as a product, the Equation 7.3 shows the specific equation for acetic acid, which is the product from the equation of the metabolic pathway that was used to calculate the theoretical yield.

Equation 7.2. General equation of hydrogen production through photodegradation of organic acids. Adapted from (Rodríguez Muñoz, 2015).

$$C_xH_yO_z + (2_x - z)H_2O \xrightarrow{Light\ energy} \left(\frac{y}{2} + 2x - z\right) H_2 + xCO_2$$

Equation 7.3. Hydrogen production through photo fermentation of acetate. Adapted from (Rodríguez Muñoz, 2015).

$$C_2H_4O_2 + 2H_2O \xrightarrow{Light\ energy} 4\ H_2 + 2\ CO_2$$

The photo fermentation process begins with the breakdown of organic acids, liberating electrons and producing carbon dioxide and NADH, with the energy in form of ATP obtained from the light during the photosynthesis, NADH reduces the ferroxin, the electrons resulted from that reduction and more photosynthetic ATP are used by the nitrogenase to reduce protos into hydrogen when nitrogen is absent, if it is present, ammonia is formed (Zhang et Zhang, 2019) (Rodríguez Muñoz, 2015) (Stephen et al., 2017). The figure 7.2 is a schematic illustration of the process described.

In the photo fermentation process a suitable light source is important, the most common ones are tungsten lamps due to the wide light spectrum they have, but it is also possible to use incandescent, fluorescent, infrared, halogen lamps and simple sun light. Also, some studies suggest that the best source to use is halogen lamps. (Rodríguez Muñoz, 2015).

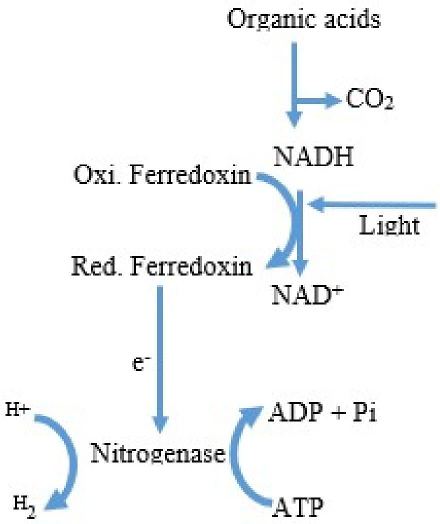

Figure 7.2. Hydrogen production through photo fermentation of organic acids. Adapted from (Zhang et Zhang, 2019) and (Rodríguez Muñoz, 2015)

Another factor that could have influenced the hydrogen production yield is the oxygen content. The three reactors were set to have anaerobic conditions, they were filled with still distilled water to the top and sealed, but anaerobic conditions can be ensured in more cautious ways. Authors like (Oliva-Rodríguez et al., 2019) and (Rodríguez Muñoz, 2015) suggest the use of nitrogen or helium gas to ensure complete anaerobic conditions. A small oxygen content in the reactors might have affected the MHPY.

(Pandey et al., 2008) reported that HPB can be affected by molecular oxygen due to the sensitivity of the enzyme Fe-only hydrogenase. (Brožová et al., 2018) state that most bacteria from the genus *Clostridium* are strict anaerobes, but they state that both the endospores and some strains can tolerate oxygen at atmospheric pressure.

(Oliva-Rodríguez et al., 2019) recommend the use of a mixed culture of the genus *Clostridium* with *Bacillus subtilis,* according to this authors, this *Bacillus* strain enhance the growth of *Clostridia* by removing any oxygen left after setting up the reactors at anaerobic conditions, the maximum hydrogen production yield is not affected much by the competition of substrate between *Clostridia* and *Bacillus*, because the latter ones are strict anaerobes, thus once the oxygen is completely depleted, these bacteria stop using the substrate and allow the *Clostridia* to grow in optimal conditions.

8 Conclusions

The objective of this study was accomplished, the hydrogen production potential of the Suchdolský Jeník brewery sludge was assessed, giving as result a small potential to produce the gas of interest at certain environmental conditions.

The proposed hypothesis must be rejected, only one of the reactors produced a volume of gas within the expected range, a volume of 145 ml, the other two reactors produced less than 100 ml, which is the lowest value in the expected range.

Additionally, none of the reactors produced 4 moles of hydrogen per mole of glucose, the reactor with the maximum gas production had a yield 35 times smaller than the theoretical one. For those two reasons, it is necessary to conclude that there is little potential to produce hydrogen under anaerobic conditions using the sludge resulting from Suchdolský Jeník brewery at the tested conditions, initial pH of 6.65. 35°C and heat shock pre-treatment at 90°C for 1 hour.

Poor environmental conditions such as iron deficiency, a sudden drop of the pH, molecular oxygen and hydrogen partial pressure might have caused a hostile environment for the hydrogen producing bacteria, this combined with a low concentration of microorganisms or endospores in the sludge might have caused that not many HPB were present in the reactors, the low presence of bacteria might be caused by a the technique used in the brewery, which involves several boils of the grain, and the difference between the reactors can be caused by a non-homogenous distribution of the microorganisms.

The pH is perhaps the most researched parameter, and authors report different ideal values, although, most researchers coincide in a value within the range of 5 and 7. Based on the results, it is suggestable to use a higher initial pH, hence a greater amount of buffer, when using Suchdolský Jeník brewery sludge at the other set conditions, e.g. temperature, amount of glucose, etc. Other buffers could be used as well, such as sodium hydroxide.

Repetitions with the resulting sludge of other brewing batches could be made to have a more statistical supported conclusion, each batch can have a different microorganism diversity and population amount after the brewing process.

Also, a characterization of the microbial biota in the sludge would be pertinent, it can either be done actively looking for the strains know to have the higher yields, such as *Clostridium acetobutylicum, Clostridium acetobutylicum ATCC 824, Clostridium butyricum CGS5, Clostridium butyricum CGS2, Clostridium pasteurianum CH4, Clostridium paraputrificum M-*

21, Clostridium thermocellum 27405, Clostridium thermolacticum, Clostridium sp. strain no. 2, Clostridium sp. Fanp2.

Another methodology would be to Gram stain samples from the sludge and only to continue isolating the gram positive bacteria and to use them as inoculum, subsequently, cultivate them in test tubes connected to a gas collector, and continue with the isolating process only with the ones that produce gas, and finally identify them and compare yields within them in order to choose the best strains from the sludge.

It is also advisable to make trials either with pure cultures, or mixed ones with those selected strains. The mixed cultures can also be made adding another genus brought from another sample from another residue, or simply bought, the external genus would act as an enhancer creating a symbiotic interaction with the strains found in the brewery's residue, such added genus could be *Bacillus*, which authors have reported to reduce the molecular oxygen content in the reactors, to facilitate *Clostridia's* growth.

If in future trials the gas produced is significant and the reactors reach a considerable efficiency, chromatography can be done to accurately evaluate the hydrogen content. Additionally, an identification of the liquid phase of the reactors could be made to corroborate that the acetate pathway was followed, this could lead to a better comparison between the stoichiometric calculations and the results form chromatography.

The current usage of the sludge produced in Suchdolský Jeník brewery is feeding the animals from surrounding farms, and considering the results, changing its usage would not be advisable, the residue has already an added value, it is beneficial to the ruminants diet, and its use has a positive economic impact in both the brewery and the farms using it, since the brewery can save resources by not having to dispose the residue on a more conventional and expensive way, and the farmers receive a low cost feed for their animals.

However, hydrogen production using this residue should not be completely dismissed , by using this sludge to do research in the hydrogen producing field, it is possible to assess the energetic value of the residue, it promotes studies in biological hydrogen production and subsequently in the technologies using this element in the energy field, not only in the production itself, which is has already efficiency difficulties, but also the storage, which is within the entirety of the energetic industry from hydrogen, the biggest complication.

The maximum hydrogen production yield was not achieved at the set environmental conditions, but it would be advisable to continue doing research in this field trying different starting conditions.

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10 List of Figures

11 List of tables

12 List of equations

13 List of Abbreviations and Symbols

ADP: Adenosine diphosphate
ATP: Adenosine triphosphate
$C_6H_{12}O_6$: Glucose
CO_2: Carbon dioxide
COH: Carbohydrates
CSS: composite storage system
Fd: Ferredoxin
H_2: Hydrogen.
HPB: Hydrogen producing bacteria.
MHPY: Maximum hydrogen producing yield.
molE: Experimental moles.
molT: Theoretical moles.
ɳ: Efficiency
n: moles of the produced gas.
NAD: Nicotinamide adenine dinucleotide (oxidized)
NADH: Nicotinamide adenine dinucleotide (reduced)
nH2: Moles of hydrogen.
nT: Total moles of the gas.
P: Pressure of the produced gas.
Patm: Atmospheric pressure.
Pg: Pressure of the produced gas.
PH_2O: Pressure of the water vapor.
Pi: inorganic phosphates.
Pi: Partial pressure.
PT: Total pressure.
R: Ideal gas constant.
T: Temperature of the gas.
V: Volume of the produced gas.
VFAs: Volatile fatty acids
Xi: Mole fraction.
Xi: Mole fraction.