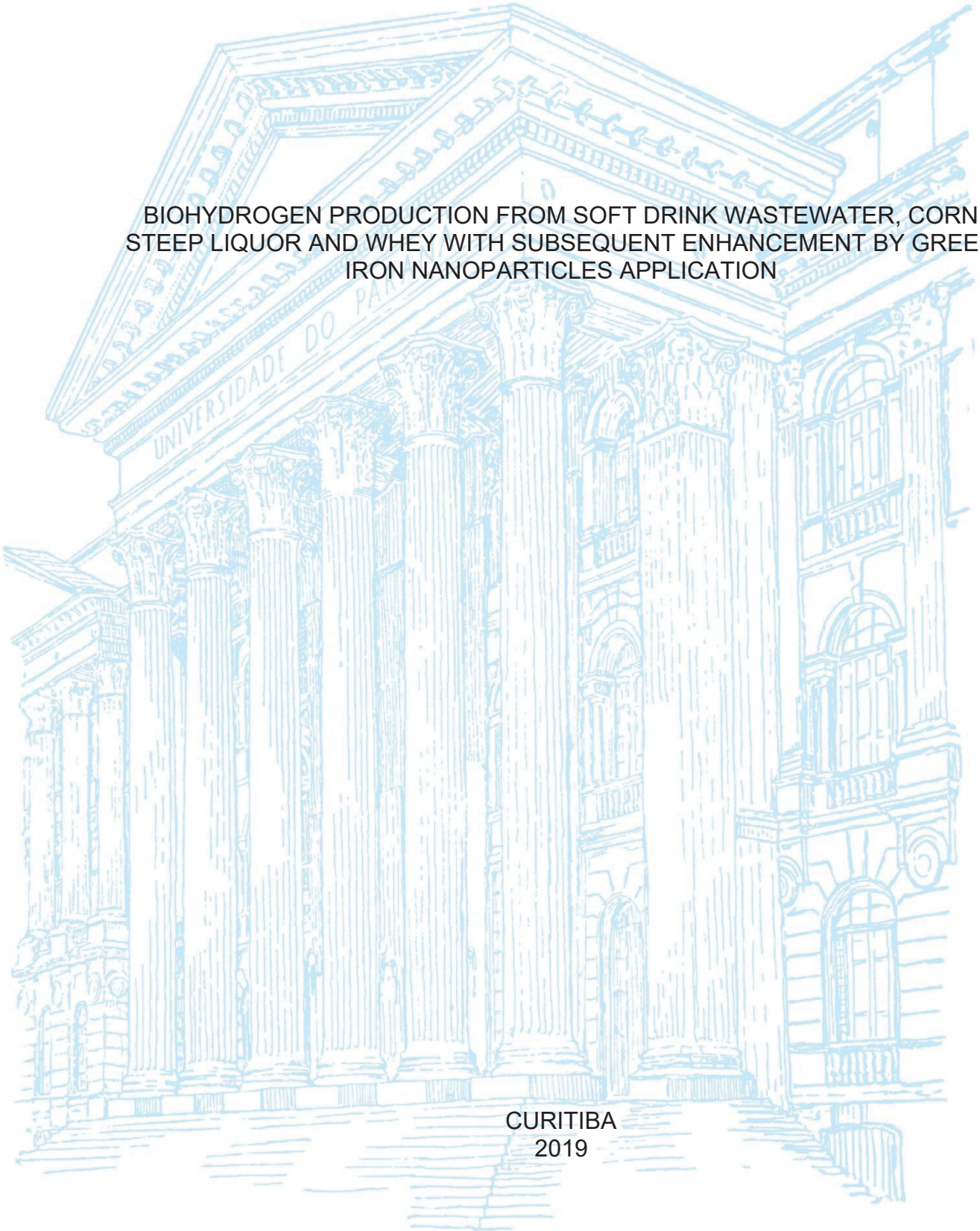
UNIVERSIDADE FEDERAL DO PARANÁ

JAIR ROSÁRIO DO NASCIMENTO JUNIOR



BIOHYDROGEN PRODUCTION FROM SOFT DRINK WASTEWATER, CORN STEEP LIQUOR AND WHEY WITH SUBSEQUENT ENHANCEMENT BY GREEN IRON NANOPARTICLES APPLICATION

CURITIBA
2019

JAIR ROSÁRIO DO NASCIMENTO JUNIOR

BIOHYDROGEN PRODUCTION FROM SOFT DRINK WASTEWATER, CORN STEEP LIQUOR AND WHEY WITH SUBSEQUENT ENHANCEMENT BY GREEN IRON NANOPARTICLES APPLICATION

Dissertação apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Engenharia de Bioprocessos de Biotecnologia.

Orientador(a): Prof(a). Dr(a). Adriane Bianchi Pedroni Medeiros

Coorientador(a): Prof(a). Dr(a). Walter José Martinez-Burgos

CURITIBA
2019

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
UNIVERSIDADE FEDERAL DO PARANÁ
SISTEMA DE BIBLIOTECAS – BIBLIOTECA DE CIÊNCIA E TECNOLOGIA

Nascimento Júnior, Jair Rosário do
Biohydrogen production from soft drink wastewater, corn steep liquor and whey with subsequent enhancement by green iron nanoparticles application / Jair Rosário do Nascimento Júnior. – Curitiba, 2019.
1 recurso on-line : PDF.

Dissertação (Mestrado) - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Adriane Bianchi Pedroni Medeiros
Coorientador: Walter José Martinez-Burgos

1. Hidrogênio. 2. Nanopartículas. 3. Fermentação. I. Universidade Federal do Paraná. II. Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia. III. Medeiros, Adriane Bianchi Pedroni. IV. Martinez-Burgos, Walter José. V. Título.

Bibliotecário: Leticia Priscila Azevedo de Sousa CRB-9/2029

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **JAIR ROSÁRIO DO NASCIMENTO JUNIOR** intitulada: **BIOHYDROGEN PRODUCTION FROM SOFT DRINK WASTEWATER, CORN STEEP LIQUOR AND WHEY WITH SUBSEQUENT ENHANCEMENT BY GREEN IRON NANOPARTICLES APPLICATION**, sob orientação da Profa. Dra. ADRIANE BIANCHI PEDRONI MEDEIROS, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 15 de Maio de 2020.

Assinatura Eletrônica
16/05/2020 11:35:58.0
ADRIANE BIANCHI PEDRONI MEDEIROS
Presidente da Banca Examinadora

Assinatura Eletrônica
15/05/2020 17:11:05.0
JULIO CESAR DE CARVALHO
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
15/05/2020 17:22:14.0
WALTER JOSE MARTINEZ BURGOS
Avaliador Interno Pós-Doc (UNIVERSIDADE FEDERAL DO PARANÁ)

Assinatura Eletrônica
15/05/2020 18:23:17.0
EDUARDO BITTENCOURT SYDNEY
Avaliador Externo (UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ)

I would like to dedicate this accomplished work to my parents, Célia and Jair, to my inseparable partner in crime, my boyfriend Douglas, to my two mentors, Professor Adriane and Walter and also to all of my friends and people who somehow believed in me throughout these difficult times for all Brazilian researchers.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my two mentors, Professor Adriane and Walter for all the guidance, advices and patience throughout this research. It has not been an easy time for researchers like us who ultimately have not had their work taken seriously in this country. People who dedicate a considerable time every single day, every week to produce scientific knowledge for Brazil. Kudos to you. Kudos to every single student who wants to be a post graduate student in this country. It is not an easy path to take.

Secondly but not less importantly, I would like to thank my partner and boyfriend for being with me in this whole process. We know how this was not easy for both of us. But it was softer because we had each other. For every morning, afternoon and night we went to university together. You drove me to the laboratory no matter the time when it was a bursting cold in the winter or when it was hot as hell in summer. For every coffee we had, for every lunch we had, for every hour you had to wait for me. You were always there. To pick me up, to advise, to study French, just to be by my side… I knew you were always going to be there. I love you unconditionally.

To my parents and family who believed in me since I started chemical engineering course. For all the emotional and financial support. For placing the bets on me and trusting in me unconditionally. I love all of you and would give my life for all of you.

I also would like to thank my lab friends and colleagues. Golden star for Leo and Jéssica who helped me a lot on pre-experiments, just to calculate media concentrations, to purge a tube or to wash them. We know how stinky they were. Amanda, Brigitte, Alexander, Elisangela, Denise, Rafaela, Carol, Zulma, Diego, Raphael, Daniely, Luis, thank you all for being part of this moment of my life. I will never forget you all. To all my professors and staff who contributed to my professional improvement and provided me know-how to pursue this work. Mitiyo, I love you!!!

Last but not least, thanks to CAPES, Vale, Ambev, Ingredion and Universidade Federal do Paraná for making this work come true.

You build a castle in the clouds
And then the fire burned you down
But you’re still standing anyway
When all you need from the start
Was waiting there inside your heart to be born again
Like a Phoenix rising from the ashes
Like a Phoenix rising from the flames
You’re gonna be alright
You’re gonna be alright again
(NELLY FURTADO, 2017, PHOENIX - THE RIDE ALBUM)

RESUMO

Duas linhagens, *Clostridium beijirinckii e Clostridium butyricum* isolado, e um consórcio LPB AH8, foram avaliados como possíveis produtores de biohidrogênio. Afim de se avaliar esses microrganismos, testes foram conduzidos com água residual da produção de refrigerantes, soro de leite e milhocina. Todos os meios foram otimizados e, particularmente, a concentração de milhocina e o tempo de fermentação foram fatores mais significativos, influenciando diretamente a fermentação escura. Todas as cepas se provaram altamente produtivas nos meios otimizados e a milhocina foi consolidada como um resíduo promissor para uma produção de biohidrogênio eficiente devido ao seu notório conteúdo nutricional que pode vir a viabilizar a produção industrial no futuro. Devido a sua composição em aminoácidos,a milhocina provou que pode ser um substituo barato da L-cisteína comercial utilizada em meios anaeróbicos. As máximas produções obtidas por *Clostridium beijirinckii, Clostridium butyricum* e o consórcio LPB AH8 foram 465 mLH_2 / g $DQO_{removida}$,1067.6 mLH_2 / g $DQO_{removida}$ and 180.72 mLH_2 / g $DQO_{removida}$, respectivamente. Nanopartículas verdes foram sintetizadas a partir de lignina com o intuito de avaliar sua influência na produção de biohidrogênio. Os maiores aumentos de produção foram obtidos com o consórcio LPB AH8, cujos rendimentos aumentaram em 2.8 e 2.3 vezes com nanopartículas de magnetita e nanopartículas de lignina não-magnéticas (LNMNP), respectivamente.

Palavras-chave: Biohidrogênio; Otimização; Milhocina; Nanopartículas verdes; Fermentação escura.

ABSTRACT

Two strains, *Clostridium beijirinckii* andan isolated *Clostridium butyricum,* and a consortium LPB AH8 were evaluated as possible biohydrogen producers. In order to evaluate these strains ability to produce biohydrogen, tests were conducted with soft drink wastewater, cheese whey and corn steep liquor. All media were optimized and particularly corn steep liquor concentration and the time of fermentation werethe most significant factors influencing dark fermentations for each strain. All strains proved to be high biohydrogen producers in optimized media and corn steep liquor was consolidated as a promising residue for biohydrogen production due to its remarkable nutritional content that may allow future industrial applications of this biogas. Due to its amino acids content, corn steep liquor proved to be a suitable replacement for commercial available L-cysteine. Maxima biohydrogen yields achieved by *Clostridium beijirinckii, Clostridium butyricum* and consortium LPB AH8 were 465 mLH_2 / g $COD_{removed}$,1067.6 mLH_2 / g $COD_{removed}$ and 180.72 mLH_2 / g $COD_{removed}$, respectively. Green nanoparticles were synthesized with lignin in order to evaluate its influence in biohydrogen production. Higher increases were obtained with the consortium LPB AH8, whose yields were increased by 2.8 and 2.3 fold with magnetite nanoparticles and lignin non-magnetic nanoparticles (LNMNP) nanoparticles, respectively.

Keywords: Biohydrogen; Optimization; Corn steep liquor; Green nanoparticles; Dark Fermentation

LIST OF FIGURES

FIGURE 1 – METABOLIC ROUTES IN BIOHYDROGEN PRODUCTION 21
FIGURE 2 - CONTOUR SURFACES 33
FIGURE 3 - ACTUAL VALUES VS PREDICTED VALUES. 34
FIGURE 4 – KINETICS OF ACCUMULATED BIOHYDROGEN VOLUME (A) AND (B) KINETICS OF SUBSTRATE DEPLETION AND PRODUCT FORMATION 36
FIGURE 5 - MAXIMUM-LIKELIHOOD TREE BASED ON 16S RRNA GENE SEQUENCES SHOWING THE PHYLOGENETIC RELATIONSHIPS. BOOTSTRAP VALUES (%) BASED ON 1000 REPLICATIONS ARE SHOWN AT BRANCH POINTS. THE SUBSTITUTION MODEL USED WAS KIMURA 2-PARAMETER MODEL BAR, 0,05% SEQUENCE DIVERGENCE 43
FIGURE 6 - CONTOUR AND SURFACE GRAPHICS OBTAINED WITH DCCR OPTIMIZATION.....45
FIGURE 7 - ACTUAL VALUES VS PREDICTED VALUES 45
FIGURE 8 - KINETICS OF ACCUMULATED BIOHYDROGEN VOLUME 47
FIGURE 9 - KINETICS OF SUBSTRATE DEPLETION AND PRODUCT FORMATION 48
FIGURE 10 – COMPARISON BETWEEN BIOHYDROGEN PRODUCTION IN MEDIA WITH AND WITHOUT CYSTEINE 49
FIGURE 11 – DETERMINATION OF AMINO ACID CONCENTRATION PRESENT IN THE LIQUID PHASE OF CORN STEEP LIQUOR 50
FIGURE 12 – AMINO ACID KINETICS PROFILE THROUGHOUT DARK FERMENTATION 51
FIGURE 13 - TEM AND EDS ANALYSES OF A) LMNP B) LNMNP 61
FIGURE 14 - A) FTIR AND B) XRD ANALYSES OF LMNP AND LNMNP 61
FIGURE 15 – CONTOUR AND SURFACE GRAPHICS OBTAINED WITH DCCR OPTIMIZATION.....62
FIGURE 16 – REGRESSION MODEL FOR THE BIOHYDROGEN PRODUCTION BY LBP AH8.....63
FIGURE 17 – ACCUMULATED BIOHYDROGEN VOLUME PRODUCED IN KINETICS 64
FIGURE 18 – KINETICS OF SUBSTRATE CONSUMPTION AND PRODUCT FORMATION.....67
FIGURE 19 - COMPARISON OF BIOHYDROGEN PRODUCTION WITH NANOPARTICLES AND THE CONTROL 68

LIST OF TABLES

TABLE 1 - PHYSICOCHEMICAL CHARACTERIZATION OF THE SUBSTRATES USED 30
TABLE 2 - BOX-BEHNKEN DRAW MATRIX 32
TABLE 3 - CHARACTERISTICS AND PARAMETERS OF THE MODEL 34
TABLE 4 - CENTRAL COMPOSITE DESIGN FOR BIOHYDROGEN PRODUCTION USING CLOSTRIDIUM BUTYRICUM 41
TABLE 5 - PHYSICAL-CHEMICAL COMPOSITION OF BW AND CSL. 55
TABLE 6 - CENTRAL COMPOSITE DESIGN FOR BIOHYDROGEN PRODUCTION SUING LPB AH8 CONSORTIUM 57
TABLE 7 - COMPARATIVE ENHANCEMENT IN BIOHYDROGEN PRODUCTION APPLYING NANOPARTICLES 69

LISTA DE ABREVIATURAS OU SIGLAS

SIGLA - Nome por extenso
SIGLA - Nome por extenso
SIGLA - Nome por extenso
SIGLA - Nome por extenso
SIGLA - Nome por extenso

LISTA DE SÍMBOLOS

© - copyright
@ - arroba
® - marca registrada
Σ - somatório de números
Π - produtório de números

SUMMARY

1 INTRODUCTION 16
1.1 JUSTIFICATION 16
1.2 OBJECTIVES 16
1.2.1 General Objective 17
1.2.2 Specific Objectives 17
2 LITERATURE REVIEW 18
2.1 ENVIRONMENTAL AND ECONOMIC ASPECTS RELATED TO BIOHYDROGEN AND ITS PRODUCTION 18
2.2 BIOHYDROGEN PRODUCING MICROORGANISMS AND THEIR METABOLIC ROUTES 19
2.2.1 Iron and hydrogenases in anaerobic bacteria metabolism 20
2.3 SOFT DRINK WASTEWATER 21
2.4 WHEY 22
2.5 CORN STEEP LIQUOR 22
2.6 EMPTY FRUIT BUNCHES 23
2.7 NANOPARTICLES APPLIED TO BIOHYDROGEN PRODUCTION 24
3 EVALUATION AND OPTIMIZATION OF SOFT DRINK WASTEWATER, CORN STEEP LIQUOR AND CHEESE WHEY MEDIA ON BIOHYDROGEN PRODUCTION BY CLOSTRIDIUM BEIJERINCKII 25
3.1 INTRODUCTION 26
3.2 MATERIALS AND METHODS 27
3.2.1 Inoculum Sampling 27
3.2.2 Characterization of wastewaters 27
3.2.3 Experimental design, optimization and statistical analyses 28
3.2.4 Kinetic profile of bioH_2 production and volatile fatty acids 29
3.3 RESULTS AND DISCUSSION 30
3.3.1 Physicochemical characterization of effluents 30
3.3.2 Variables selection and optimization 31
3.3.3 Mathematical model 33
3.3.4 Evaluation of bioH2 production in optimal conditions 34
3.4 CONCLUSION 36
4 EVALUATION OF AMINO ACIDS KINETICS IN BIOHYDROGEN PRODUCTION FROM SOFT DRINK'S WASTEWATER AND CORN STEEP LIQUOR BY CLOSTRIDIUM BUTYRICUM 37
4.1 INTRODUCTION 37
4.2 MATERIAL AND METHODS 38
4.2.1 Inoculum Sampling 38
4.2.2 Characterization of wastewaters 40
4.2.3 Experimental design, optimization and statistical analyses 40
4.2.4 Kinetic profile of bioH_2 production and volatile fatty acids 42
4.2.5 Evaluation of amino acids and presence/absence of cysteine in media 43
4.3 RESULTS AND DISCUSSION 43
4.3.1 *Clostridium butyricum* isolation 43
4.3.2 Variables selection and optimization 44
4.3.3 Evaluation of kinetic profile of biohydrogen production and volatile fatty acids 46
4.3.4 Amino acids evaluation in medium 48
4.4 CONCLUSION 52

5 ENHANCEMENT OF BIOHYDROGEN PRODUCTION IN INDUSTRIAL WASTEWATERS WITH VINASSE POND CONSORTIUM USING GREEN IRON NANOPARTICLES 53
5.1 INTRODUCTION 53
5.2 MATERIALS AND METHODS 54
5.2.1 Inoculum sampling 54
5.2.2 Characterization of residues 55
5.2.3 Experimental design, optimization and statistical analyses 56
5.2.4 Kinetic profile of biohydrogen production and volatile fatty acids 57
5.2.5 Lignin extraction from oil palm empty fruit bunches 58
5.2.6 Green iron nanoparticles synthesis 59
5.2.7 Nanoparticles characterization 59
5.3 RESULTS AND DISCUSSION 60
5.3.1 Iron nanoparticles characterization 60
5.3.2 Optimization 61
5.3.3 Kinetic profile of biohydrogen production and volatile fatty acids 63
5.3.4 Evaluation of the presence of nanoparticles in media 67
5.4 CONCLUSIONS 71
REFERENCES 71

1 INTRODUCTION

Biohydrogen is universally accepted as an alternative renewable energy source since its combustion as a fuel does not generate greenhouse effect gases and, due to the reduction in environmental impact in general, this gas is considered ideal and a clean substitute for fossil fuels.

1.1 JUSTIFICATION

The production of biohydrogen and the whole concept of bioenergy can be related to the use of industrial waste and sources of renewable biomass (Mishra et al., 2017). Many researchers have been looking for renewable energy sources that can replace fossil fuels due to climate change that is plaguing the entire world today. In addition, the decrease in the stock of non-renewable energy sources, such as oil and coal, is critical due to their indiscriminate use (Li et al., 2012).

Given the problems involved in relation to the scarcity of non-renewable energy sources, the environmental impacts caused by its massive use and the overload of waste disposal due to industrial and domestic practices in the environment, the search for technologies called “green” are very promising and has been the focus of many researchers, such as the development of sustainable biorefineries. The biorefinery itself has invested in producing biofuels, as well as fine chemicals and also energy from renewable sources, such as biomass (Isla et al., 2013).

The production of hydrogen by dark fermentation has been considered an excellent alternative for the generation of clean energy. To this end, it is also aimed at recycling a variety of industrial wastes, such as effluents with a high organic load, which, in parallel with the production of value-added compounds, are also treated, having their polluting power decreased (Yang; Wang 2018). Since its combustion does not generate polluting gases and because it is an excellent carrier of energy, the biohydrogen produced from wastewater has been studied and explored, especially when the production is given by the dark fermentation route (Sivagurunathan et al., 2015; Kothari et al., 2012).

1.2 OBJECTIVES

1.2.1 General Objective

The general objective of this work is to produce biohydrogen from industrial waste and to verify the influence of the introduction of iron nanoparticles in the fermentation media.

1.2.2 Specific Objectives

The specific objectives of this work are:

a) to characterize the residues used in fermentation processes;
b) to test strains previously known for producing biohydrogen;
c) to carry out experimental planning and optimize the fermentation media for biohydrogen production;
d) to evaluate the kinetics of biohydrogen production and volatile fatty acids;
e) to produce and apply green iron nanoparticles in the fermentation media in order to verify their influences on biohydrogen production;
f) to verify the influence of L-cysteine absence in media;
g) to verify the behavior of amino acids consumption by *Clostridium butyricum*.

2 LITERATURE REVIEW

2.1 ENVIRONMENTAL AND ECONOMIC ASPECTS RELATED TO BIOHYDROGEN AND ITS PRODUCTION

Given the huge amount of wastewater from the food industry, pollution of river waters, due to the inappropriate disposal of these wastes, takes on an extremely important proportion today (Liu et al., 2015a). As much as all residual water from the production of soft drinks and other industries have a variant composition from industry to industry, these effluents present a concrete risk to the environment and to human life due to the impact on groundwater water quality caused by infiltration or indirect disposal. The organic load discharged into rivers by industrial effluents decreases the amount of dissolved oxygen directly impacting the survival of fish and other organisms, since an oxygen level less than 2 $mg.L^{-1}$ inside the cells causes cellular malfunction and impairs the circulatory fluid balance which may cause the death of aquatic species. The presence of phosphorus and nitrogen in the waste affects the quality of the water. Basically, an inappropriate disposal would cause water eutrophication (Hidalgo & Martín-marroquín, 2019).

Products used in soft drinks, such as fungicidal sorbates, are ecologically toxic. Given the need to treat industrial effluents, the control and treatment of water from the soft drink industry has a major challenge ahead, especially when adding value to its productivity chain with sustainability (Hidalgo & Martín-marroquín, 2019)).

A considerable amount of the world energetic matrix is supplied by fossil fuels like oil, coal and natural gas which are responsible for the increase of greenhouse gases in atmosphere. Hydrogen emerge as a clean energy alternative to these current fuels because its combustion reduce the pollution in atmosphere since only water generated (Lee et al., 2011). To become sustainable though, it is necessary to find other ways of producing hydrogen once its existing methods are highly energy demanding, consequently, involving harsh thermochemical processes defined by coal gasification, hydrocarbon reforming (steam reforming), partial oxidation of hydrocarbons and water electrolysis (Bittencourt et al., 2014; Lee et al., 2011; Said et al. (2017).

Once the chemical process conditions for obtaining hydrogen are so extreme, the costs on maintenance of these processes are also high. Recently, attention has been given to develop an economic process for producing biological hydrogen or

biohydrogen with energy yield of 122 kJ / g, 2.75 folds greater compared to fossil fuels (Kamalaskar et al., 2016). Fermentative biohydrogen production can be combined to industrial waste treatments such as soft drink wastewater and corn steep liquor to reduce the economic aspects of biohydrogen and also reuse these residues for energy recovery. By reutilizing these low-value biomass and wastewater in parallel with high yields biohydrogen producing microorganisms, it is possible to enable dark fermentation in a sustainable economic way since it does not require high energy input or light (Pattra et al., 2008; Said et al., 2017; Seppa, 2011; Zhao et al., 2011). Energy factors combined with reducing the main limiting costs of dark fermentation, such as feedstock, allows the scaling up of the process to further commercializing (Hamilton et al., 2018).

2.2 BIOHYDROGEN PRODUCING MICROORGANISMS AND THEIR METABOLIC ROUTES

Biohydrogen-producing microorganisms are merged into three groups: facultative, strict, and photosynthetic anaerobic microorganisms. Photosynthetic microorganisms (*Rhodobacter* and *Rhodopseudomonas*) are known for degrading volatile fatty acids to produce biohydrogen. Photo-fermentative biohydrogen production reaches high production yields with high conversion of substrate in biohydrogen, but, although this kind of fermentation is related to high yields it only prevails in the presence of light. Photo-fermentation is also challenged by the lack of efficient bioreactors and cheap raw materials that would enable photo-biohydrogen production (Liu et al., 2017; Nasr et al., 2015; Pandey et al., 2014).

Anaerobic bacteria involved in dark fermentation are more attractive for the study of biohydrogen production since the growth of bacteria is faster, there is no need for light and the instrumentation necessary for the process is minimal. Microorganisms of the genus Clostridium sp. are generally used and also the most found in consortia used for the production of biohydrogen. The presence of these species is essential, since they are considered key producers of biohydrogen. Among them it is worth mentioning *Clostridium butyricum, Clostridium beijerinckii, Clostridium acetobutylicum, Clostridium saccharoperbutylacetonicum* and *Clostridium pasteurianum* (Pattra et al., 2008; Wang et al., 2009). These species of Clostridium are known for their ability to sporulate and convert hexoses into hydrogen with a yield of 4 molH2 / mol hexose or of 2 molH2 / mol of hexose if the routes adopted are the acetate and butyrate as a final

metabolite, respectively. These yields are higher than other hydrogen producers such as bacteria of the species Enterobacter sp that produce a maximum of 1 molH2 / mol hexose. Generally, facultative anaerobes reach lower yields of biohydrogen due to metabolic limitations. (Kapdan & Kargi, 2006; Lin, P. et al., 2007).

Theoretically, the complete conversion of glucose would result in 12 mol of hydrogen but, evolutionary, microorganisms have developed to shift the metabolic pathways in majority to cell growth and maintenance. Also, in addition to biohydrogen it is also produced volatile fatty acids and alcohols. Practically, there is a combination of both acetate (1) and butyrate (2) route which is associate to high biohydrogen yields (Cai et al., 2010). Formation of biohydrogen are described below.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \textbf{(1)}$$

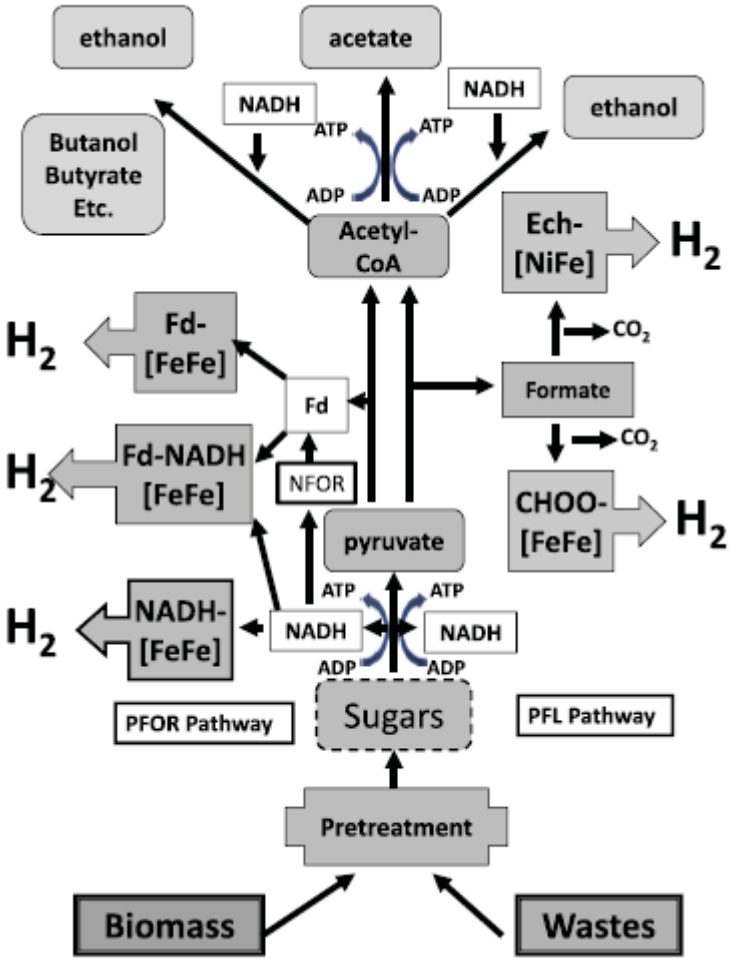
$$C_6H_{12}O_6 \rightarrow CH_3\ CH_2\ CH_2COOH + 2CO_2 + 2H_2 \quad \textbf{(2)}$$

2.2.1 Iron and hydrogenases in anaerobic bacteria metabolism

Directly related to the production of biohydrogen in many species of Clostridium, the [FeFe]-hydrogenases have an enzymatic function which is indispensable for biohydrogen production. Hydrogenase concentrations peak within cells before hydrogen production begins and before cells reach their maximum growth. Biohydrogen is produced by two different pathways (FIGURE 1) where carbohydrates can be consumed producing pyruvate which is later converted to acetyl coenzyme A. Both pyruvate and acetyl coenzyme A can be reduced by pyruvate-ferredoxin oxidoreductase (PFOR) or by pyruvate-formate lyase (PFL). Microorganisms are limited to produce only 2 mol of biohydrogen once they are driven by PFL pathway because they are not able to assimilate NADH for the formation of more biohydrogen. NADH may be oxidized if microorganisms take pathway PFOR, since this metabolic route allows [FeFe] hydrogenases not only to promote this oxidization but also accelerate this process (Hallenbeck et al., 2012).

Basically, biohydrogen production is carried through pyruvate-ferredoxin oxidoreductase (PFOR) and in small amounts through NADH-ferredoxin oxidoreductase (NFOR). These enzymatic complexes transfer electrons to [FeFe]-hydrogenase which is responsible for biohydrogen production by a reversible reduction of protons accumulated throughout fermentation (Hamilton et al., 2017)

FIGURE 1 – METABOLIC ROUTES IN BIOHYDROGEN PRODUCTION



SOURCE: Hallenbeck et al. (2012).

2.3 SOFT DRINK WASTEWATER

Water consumption is one of the most influential factors in the cost of a beverage industry. The soft drink production process contaminates an enormous amount of water with sugars and other biodegradable substances. Given the amount of sugars present in this effluent, it is possible to generate biohydrogen from dark fermentation because the sugars present in the production of soft drinks are the most rapidly assimilated by microorganisms, such as glucose and fructose (Abdel-fatah et al., 2017; Peixoto et al., 2011).

The production of biogas from industrial soft drink wastewater has already been carried out by several authors. The composition of this effluent has also been applied in the production of volatile fatty acids, bioethanol, polymers and even in the

denitrification and improvement of biological phosphorus removal. In addition to the use of residual washing water, several liters of soft drink are also discarded by these industries as they do not pass the quality control required by legislation or also by expiration date. With this, there is even more volume of waste discarded comprising of about 10 to 12% w / v of sugars available (Isla et al., 2013; Vergine et al., 2015).

2.4 WHEY

The dairy industry has a major impact on the economy in a large part of the agro-industrial sector. Whey is a saline effluent with high acidity and high organic load whose composition is extremely variable according to the conditions of the raw milk used in the process, different dilutions of milk, washing water after washing tanks, process equipment. This effluent is generated in a volume four times (approximately 90%) greater than the processed milk itself. Therefore, it is a great challenge for small and medium-sized companies to be able to dispose of this waste without their own treatment station (Carvalho et al., 2013; Escalante et al., 2018).

There are two types of whey produced in cheese industries, whey obtained from the first production of cheese, a raw cheese whey with a high nutritional load, which is reused for the production of cottage cheese, and whey “Weak”, from a washing line resulting from washing pipes, tanks and other equipment (Gioannis et al., 2014).

Several studies have been carried out in the quest to produce biohydrogen from whey. Leo et al. (2008) produced biohydrogen at a rate of 8.1 mmolH_2 / L.h with 25 g of serum powder per liter of medium at a pH equal to 7.5. Kopsahelis & Kornaros (2016) managed to obtain a productivity of 0.76 LH_2 / L.d using whey and Moreno et al. (2015) obtained 0.18 LH_2 / L.d. This proves that whey is a waste with potential for the production of biohydrogen.

2.5 CORN STEEP LIQUOR

Corn Steep Liquor (CSL) is a co-product of the corn milling industry and is basically composed of all corn water-soluble components. The term steep means accentuated, that is, the concentrate of components remaining from a “wet corn” milling, justifying its characteristics of viscous liquid and high organic load (Li et al., 2016; Xiao et al. (2013). In addition to viscosity, millet is also composed of two phases, a liquid and a solid, insoluble in water (Hofer et al., (2018).

Due to its characteristics, CSL has been used to replace yeast extract as a source of nitrogen for the production of several compounds such as lactic acid, succinic acid and ethanol, significantly reducing the cost of production (Li et al., 2016). In fermentations, it is of great importance to evaluate costs, both operational and raw materials (carbon and nitrogen), since cost reductions can via a process viable (Xiao et al., 2013; Yang et al. (2018).

The massive production of the corn starch industry has achieved rapid development and, as a result, huge quantities of CSL have been produced. Many producers end up discarding this “waste” in rivers, causing a huge environmental impact due to the high organic load of millet, consequently characterizing it as a high COD waste (Li et al., 2016).

Corn Steep Liquor is a rich source of nitrogen that contains vitamins, amino acids, minerals and many other components that stimulate the growth of microorganisms. One of the great applications of CSL due to its great nutritional source has been happening in the production of antibiotics by *Penicilium chrysogenum* (Hofer et al., 2018; Yang et al., 2013).

2.6 EMPTY FRUIT BUNCHES

The oil palm industry is the main generator of biomass which can be used as renewable sources. Empty fruit bunches or EFB is a solid residue from palm oil production. Due to the massive and increasing volume of palm oil production worldwide, especially Malaysia and Indonesia, EFB has become a huge lignocellulosic biomass source. This biomass is composed of lignin, cellulose and hemicelluloses. Lignin has a complex structure that limits enzymatic digestibility decreasing and/or detains hemicellulose and cellulose hydrolysis as lignin is associated to hemicellulose. All these complexities make lignin hard to break than cellulose or hemicellulose. Generally, EFB is composed of 45 % celllose and 25% hemicellulose which generally are liberated from lignin structure through physical-chemical such as acidic or alkaline hydrolysis and through biological methods carried by the enzymatic activity of microorganisms (Kusmardini et al., 2016; Loh, 2017).

According to Kim et al. (2018), lignocellulosic biomass sources as EFB are cost-effective and also environment friendly. This could an alternative as renewable energy source compared to fossil fuels reducing gas emissions.

2.7 NANOPARTICLES APPLIED TO BIOHYDROGEN PRODUCTION

The use of nanotechnology has become widespread in areas involving research & development such as modern science. Application of nanoparticles has been made in biomedicine, foods and beverage, cosmetics, catalysis and also in bioenergy studies. Environmental applications aiming to revert and minimize the effects of pollution caused by wastewater from industry disposal on river waters and headwaters are still being tested. Beverage wastewater poses a threat to water quality if discarded without any treatment since it is responsible for decreasing oxygen availability, impacting cellular functioning of fish and other living organisms (Esakkimuthu et al. 2017; Zhang & Shen, 2007; Hidalgo & Martín-marroquín, 2019).

Natural metabolism of certain microorganisms has great influence on the limited biohydrogen production, some of which may convert only a third of substrate available in biohydrogen, while the other two thirds are responsible for the production of volatile fatty acids from fermentation. These limitations are not an exception when it comes to dark fermentation, reason why nanoparticles and their unique characteristics have been introduced into these processes to enhance biohydrogen production through high catalytic activities promoted by the high surface area from these nanocatalysts. Properties related to nanoparticles have been associated to bioactivity increase and yield enhancement in dark fermentation (Lin et al., 2016; Taherdanak et al., 2015b; Zhao et al., 2013).

Conventional methods for synthesis of iron nanoparticles require the use of harmful chemicals as reducing agents which are not biodegradable and are potentially toxic for humans and biological systems (Bishnoi et al., 2017). Green synthesis is an alternative and environmentally friendly technique for the synthesis of nanoparticles that has made use of many plant extracts such as green tea or eucalyptus leaf (Fazlzadeh et al., 2017). Thus, lignin, a natural polymer extracted from lignocellulosic biomass that exhibits antioxidant properties, acts inhibiting the oxidation of Fe^{2+} ions by air in the co-precipitation process of synthesis of Fe_3O_4, eliminating the use of inert atmosphere required to prevent air oxidation of Fe^{2+} (Singh et al., 2017).

To ensure that biological systems work properly, small amounts of metals such as iron, copper, sodium, nickel, among others are required and their presence in the media is indispensable. There is a wide variety of metals which can be used in dark fermentation however iron is interesting due to its low cost and its capability to promote

more favorable conditions for the catalysis of proton reduction in hydrogen molecules through enzymatic activity of hydrogenases. Iron nanoparticles assist on the acceleration of electron transference between ferredoxin and hydrogenases but they also may have an inhibitory effect on microorganisms by toxicity when supplemented in high concentrations, decreasing enzymatic activities (Malik et al., 2014; Taherdanak et al. 2015; Yang & Wang, 2018).

3 EVALUATION AND OPTIMIZATION OF SOFT DRINK WASTEWATER, CORN STEEP LIQUOR AND CHEESE WHEY MEDIA ON BIOHYDROGEN PRODUCTION BY CLOSTRIDIUM BEIJERINCKII

3.1 INTRODUCTION

In the last decades, the search for alternative renewable energies has become the focus of research involving fuels. Highly polluting, the fossil fuels have started to open space to more widespread studies in search of sustainable biofuels with reduced environmental impacting (MARBÁN & VALDÉS-SOLÍS, 2007; LAY et al., 2019). Among these environmentally friendly sources of energy is hydrogen, since the gases from its combustion do not generate greenhouse effect gases, such as CO_2. Generating only water and also obtaining a high energy efficiency, hydrogen is considered a promising alternative for the new world energetic matrix (Beckers et al., 2013; Kamalaskar et al., 2016; Pattra et al., 2008).

Currently, most of the hydrogen produced is derived from processes using fossil fuels, high temperatures and pressures, which makes it necessary to search for a more sustainable production method (Hitit et al., 2017; Holladay et al., 2009). In this context, biohydrogen from microorganisms appears to be a promising option since they have the ability to consume substrates of different types and convert its organic content into hydrogen through electron transfer, without generating pollutants or high costs on extreme chemical processes (Beckers et al., 2013; Hamilton et al., 2018).

Several types of microorganisms are capable of producing biohydrogen, such as cyanobacteria, algae, aerobic and anaerobic bacteria. Producing genres involve *Bacillus, Citrobacter, Enterobacter, Escherichia* and *Rhodopseudomonas* (Nath & Das, 2004; Pan et al., 2008a). However, among the producers, the *Clostridium* genus deserves a highlight due to the highest hydrogen yields when compared to other genera and its tendency to dominate dark fermentation in cases of co-culture (Wang et al., 2009; Ye et al., 2012). Many species of this genus are capable of producing biohydrogen, such as *C. acetobutylicum, C. butyricum, C. beijerinckii* and *C. tyrobutyricum* (Lin, P. Y. et al., (2007).

The fact that these bacteria can use agro-industrial residues as a substrate for their growth is also an advantage, because in addition to being inexpensive and produced in large quantities, it allows them to be destined in a more appropriate way. Recent experiments prove that the use of substrates such as cassava processing wastewater, corn steep liquor (CSL), beverage wastewater, mushroom farm waste and other kinds of waste are suitable for biohydrogen production in significant quantities

(Kumar et al., 2015; Martinez-Burgos, W. et al., 2019; Prabakar et al., 2018; Wu et al., 2017).

Based on this information, the objective of this work was to characterize four agro-industrial residues, soft drink wastewater, corn steep liquor, cheese whey and expired Guaraná soft drink for further testing and evaluating their potential for hydrogen production using a strain of the species *C. beijerinckii*.

3.2 MATERIAL AND METHODS

3.2.1 Inoculum Sampling

Clostridium beijerinckii ATCC 8260 was submitted to tests in order to evaluate its ability of producing biohydrogen from a combination of industrial wastewaters. Firstly, the strain was reactivated in a vinasse based liquid medium where were added: (5 g / L) glucose, (1.0 g / L) $NaHCO_3$ and (0.5 g / L) cysteine-HCl at 85 ºC and 65 ºC, respectively. Upon reactivation, *Clostridium beijerinckii* was then inoculated into a pre optimization medium based of soft drink wastewater composed of: 3% CSL (v / v), (0.5 g / L) cysteine-HCl and (1.0 g / L) $NaHCO_3$ which was continuously used for maintenance of the strain at 37 ºC for 48 h with further transfer to fresh medium at each 48h. Inoculum rate adopted was 10%.

Procedures according to BALCH et al. (1979) were adopted to guarantee an anaerobic environment. Oxygen was removed from the medium by boiling (100-105°C) under anoxic conditions under an argon atmosphere. Reduction of redox potential was ensured by the addition of $NaHCO_3$ (1.0 g/L) and Cysteine-HCl (0.5 g/L) at 85ºC and 65ºC, respectively. All experiments were conducted in Hungate tubes with a working volume of 6 mL. The tubes were sealed with bakelite caps and autoclavable rubber stoppers. The pH of the medium was adjusted to 7.0 using 35% NaOH solution.

3.2.2 Characterization of wastewaters

Soft drink wastewater (SDW) and expired Guaraná provided by the company Ambev (Almirante Tamandaré, Paraná, Brazil), cheese whey (CW) provided by the company Anila (Fernandes Pinheiro, Paraná, Brazil) and corn steep liquor (CSL) provided by the company Ingredion (Balsa Nova, Paraná, Brazil) were evaluated as promising substrates biohydrogen production through dark fermentation. The industrial wastewaters were stored at -20 °C for later use. Initially, these effluents were submitted

to a physic-chemical characterization and sugar contents were determined by High Performance Liquid Chromatography (HPLC).

Cations and anions were analyzed by ion chromatography (761 Compact IC, Metrohm AG). Metrosep C 3250/4.0 and Metrosep A Supp 5 - 250/4.0 columns were used for cations and anions, respectively. Concerning the cations, mobile phase was HNO_3 3.5 mM at a flow rate of 0.9 mL/min. As for the anions, mobile phase was Na_2CO_3 3.2 mM e $NaHCO_3$ 1.0 mM at a flow rate of 0.7 mL/min. Run times were 25 and 30 min for cations and anions, respectively.

The determination of the Chemical Oxygen Demand and the total nitrogen content (COD) were done using Standard Methods for the Examination of Water and Wastewater (1992).

3.2.3 Experimental design, optimization and statistical analyses

Six variables were evaluated, namely: concentration of EFB (-1;1) where -1 means absence and 1 means it was added in medium in a concentration of (5 g / L), the fermentation time (-1;1) where -1 corresponds to 24h and 1 corresponds to 48h of fermentation time, the presence of expired soft drink (-1;1) where -1 means absence and 1 means it was added in 20% (v / v), the percentage of inoculum (-1;1) where -1 means inoculum concentration of 10% and 1 corresponds to 20% (v / v). The concentration of CSL (-1;1) where -1 means absence and 1 means 10% (v / v) and cheese whey (-1;1) where -1 means absence and 1 corresponds to 40% (v / v) were evaluated in an attempt to determine the best source of nitrogen. This planning was carried out with 6 factors and 12 runs, one for each selected strain. The Pareto diagram was used to determine the effects of significance in the experiments performed.

For *Clostridium beijerinckii*, upon significant effects determination, a Box-Behnken (BB) optimization planning with three factors, 3 central points and 15 runs was used to optimize the selected variables. The optimization provided an adjusted quadratic model equation shown below.

$$H2 = \beta o + \sum_{i=1}^{k} \beta i Xi + \sum_{i=1}^{k} \beta ii XiXi + \sum_{i=1} K - 1 + \sum_{j=1+i}^{k} \beta ij XiXj$$

This Equation provides the constant, linear, squared and interactive coefficients of the parameters inputted for optimization, which are β0, βi, βii and βij, respectively. The three independent variables (i=1-3) are represented by: $\mathbf{X_1}$ equals CSL concentration; $\mathbf{X_2}$ equals time and $\mathbf{X_3}$ equals CW concentration. H_2 is the accumulated production of bioH_2. All statiscal analysis and surface responses were obtained in the software Minitab (15). Except for the linear terms, all non-significant terms obtained from the statiscal analysis were discarded.

3.2.4 Kinetic profile of bioH_2 production and volatile fatty acids

Clostridium beijerinckii strain had the medium optimized with 14% of CSL and 45% of whey. The missing media component is residual water from soft drink production. The media were prepared according to the compositions presented above with the addition of NaHCO3 (1.0 g / L) and cysteine-HCl (0.5 g / L) at 85 ºC and 65 ºC, respectively. All experiments were conducted in Hungate tubes with a working volume of 6 mL. The tubes were sealed with bakelite caps and autoclavable rubber stoppers. The pH of the medium was adjusted to 7.0 using 35% NaOH solution. The inoculum rate used was 10% and culture media were incubated at 37 ºC upon inoculation.

Analysis of biohydrogen production and fatty acids was conducted periodically at 16h, 32h, 48h, 72h and 96h. A glass syringe was used to collect the produced gas which was further analyzed in a 490 Micro GC System gas chromatograph (Agilent), equipped with two columns (Molsieve 5Å and PoraPLOT U) and a thermal conductivity detector (μTCD). In the Molsieve 5Å column, the injection temperature was 110 ° C with an injection time of 20 ms, column temperature of 90 ° C and an initial pressure of 190 kPa. In the PoraPLOT U (PPU) column, the injection temperature was 110 ° C with a column temperature of 90 ° C and an initial pressure of 150 kPa. The peak retention time for biohydrogen was 0.4 min (Ms5A) and for CO2 and CH4 were 0.405 and 0.425 respectively in the PPU. The time for each run was 1.2 min. The mobile phase used was argon gas with a purity of 99.999%.

The composition of fatty acids produced and substrate depletion was analyzed using HPLC (High Performance Liquid Chromatography) at the same times where the biohydrogen samples were analyzed. Prior to injection of the samples (1mL), they were submitted to centrifugation at 6000 rpm for 10 min and microfiltration in cellulose

acetate membranes (0.22 µm pore). The chromatograph used was an Agilent 1260 Infinity Quaternary LC with RI detector and Hi-Plex-H column. The detector and column temperature were 50 ° C and 60 ° C, respectively. The mobile phase used was 5mM H2SO4 at a flow rate of 0.6mL / min and the injection volume was 10 µL.

3.3 RESULTS AND DISCUSSION

3.3.1 Physicochemical characterization of effluents

It was observed that the three residues or effluents are composed of macro and micronutrients (TABLE 1), which can be used in microbial growth and in obtaining energy in the form of biogas, mainly hydrogen. It is noteworthy that the main carbon sources of the effluents are simple carbohydrates (mono and disaccharides) which are easily accessed by microorganisms, being this a characteristic advantage of these residues once additional processes are not necessary to increase their bioavailability. Previous works have already shown that these effluents contain significant amounts of carbon sources easily assimilated by microorganisms (Alvarado-Cuevas et al., 2013; Hofer et al., 2018; Wickham et al., 2018). In addition, CSL also contains high amounts of lactic acid, which can be used as an alternative carbon source in the production of biohydrogen (Martinez-Burgos et al., 2020). The high quantity of organic matter in the effluents justifies their high chemical oxygen demand. Previous work has also shown that CSL and whey effluents contain high COD (Farizoglu et al., 2004; Zhang et al., 2009), needing treatment before being disposed of in the environment.

The effluents also contain significant amounts of nitrogen, mainly CSL and Whey, which makes them attractive for fermentation processes, since they are low-cost by-products when compared to other sources of nitrogen, such as yeast extract, meat extract, ammonium sulfate, among others Alvarado-Cuevas et al. (2013) & Maddipati et al. (2011). Also, these residues are a source of micronutrients, such as Ca, Mg, Cl, F and other elements necessary for the growth of microorganisms.

TABLE 1 - PHYSICOCHEMICAL CHARACTERIZATION OF THE SUBSTRATES COMPONENTS USED TO PREPARE THE FERMENTATION MEDIA

Parameters	CSL	Soft Drink Wastewater	Whey
K (mg L^{-1})	38,000	38.3	1,650
Fe (mg L^{-1})	<100	3.0	-
Mn (mg L^{-1})	60.0	-	-
Cu (mg L^{-1})	47.0	-	-
Zn (mg L^{-1})	60.0	-	-
Na(mg L^{-1})	1,412	268.6	805.0

Ca (mg L^{-1})	1,170	43.0	819.0
Mg (mg L^{-1})	2,875	32.45	131.0
NH_4 (mg L^{-1})	1,412	5.85	217.0
NO_3 (mg L^{-1})	-	16.4	-
PO_4 (mg L^{-1})	-	-	-
F (mg L^{-1})	25.057	-	-
Cl (mg L^{-1})	36.576	87.8	-
Citric Acid (g L^{-1})	-	1.182	-
Fructose (g L^{-1})	35.49	3.998	-
Lactic Acid (g L^{-1})	92.55	0.5676	
Acetic Acid (g L^{-1})	0.3617	0.3071	-
Maltose (g L^{-1})	1.493	-	-
Glucose (g L^{-1})	36.31	0.7	-
Lactose (g L^{-1})	-	-	4.55
Nitrogen (g L^{-1})	10.8*	0.5	3,6
Phosphorus P_2O_5 (g L^{-1})	<1.0*	<1.0	12
pH	4.32	7.56	4.76
COD (g L^{-1})	245.4	4.27	47.3
COD (g L^{-1})		39.52**	Final COD 32.30**

*Concentration given in mg.g^{-1}

Source: The author, (2020)

3.3.2 Variables selection and optimization

The Pareto diagram of PB (Figure not shown) showed that, of the five variables tested, only two, % v/v CSL and time, significantly affect the production of hydrogen ($p \leq 0.05$). Although whey concentration was not significant, it was chosen to continue to use the residue since it almost reached the line of significance in the Pareto Diagram. Previous works demonstrate that the concentration of CSL, whey and the fermentation time affect hydrogen production via dark fermentation (Dçbowski et al., 2014; Ferchichi et al., 2005; Thanwised et al. (2012). However, unlike other studies (Martinez-Burgos et al., 2020; Martinez-Burgos, W. J. et al., 2019) the inoculum rate was not significant ($p \leq 0.05$), which could have happened due to the fact that similar inoculum volumes were used.

It was expected that, due to its huge amount sugars, that expired soft drink would be significant in all tests performed. Soft drinks were added to the medium in a concentration of 20% and even though it was diluted, probably, the finished product antimicrobial properties were still outstanding. According to Kregiel (2015), soft drinks contain chemical preservatives and also acids that play an important role in avoiding microbial growth. Sorbates and benzoates present in the soft drink act together to enhance antimicrobial effectiveness against bacteria and other microorganisms. Notwithstanding, the author still emphasizes that this combination cause inhibition of amino acids uptake and destroys the internal proton level of microbial cells.

Ogueri&Vincent (2017) demonstrated that contamination in soft drink's manufacturing generally thrives on process equipment but not on finished products due to its preservatives plus low pH and carbon dioxide presence.

The effect of significant variables was evaluated using a Box-Behnken design. The matrix of the experimental planning is presented in TABLE 2. 1

TABLE 2 - BOX-BEHNKEN DRAW MATRIX FOR THE OPTIMIZATION OF MEDIA USED BY CLOSTRIDIUM BEIJERINCKII

Run	CSL (%)		t (h)		Whey (%)		V H_2 (mL)
	Code X_1	X_1	Code X_2	X_2	Code X_3	X_3	
1	-1	6	1	48	0	40	4.87
2	0	10	0	36	0	40	11.35
3	1	14	0	36	-1	35	13.23
4	-1	6	0	36	-1	35	7.65
5	1	14	-1	24	0	40	5.91
6	0	10	-1	24	-1	35	6.90
7	0	10	1	48	-1	35	12.95
8	1	14	0	36	1	45	16.41
9	0	10	1	48	1	45	10.32
10	-1	6	-1	24	0	40	3.78
11	0	10	-1	24	1	45	8.10
12	-1	6	0	36	1	45	6.86
13	0	10	0	36	0	40	12.33
14	0	10	0	36	0	40	12.22
15	1	14	1	48	0	40	17.14

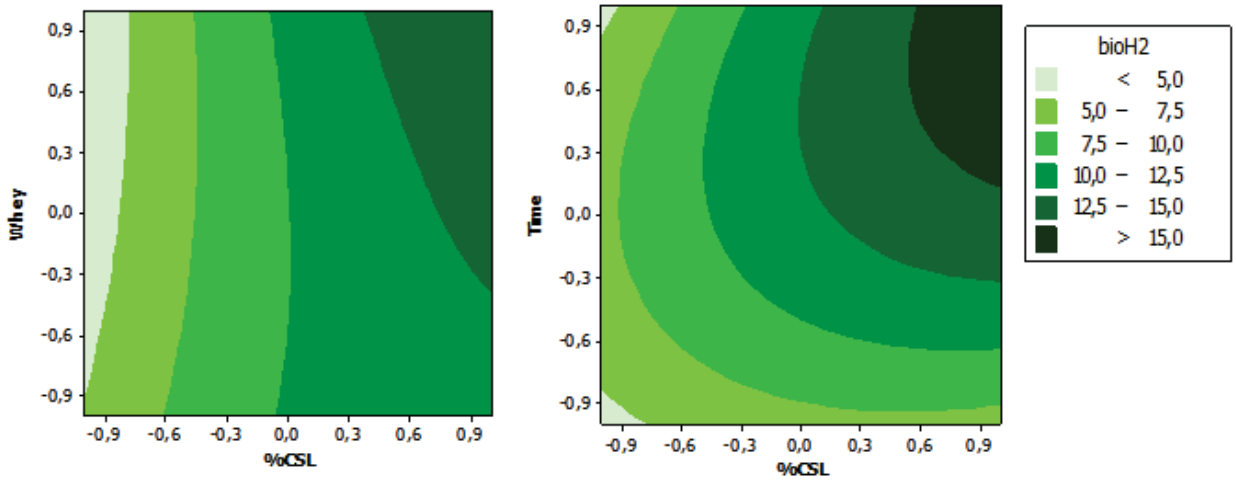
Source: The author (2020).

The contour surface graphs (FIGURE 2) show that the optimal hydrogen production region is in the intervals (12-14%), (37.5-48 h) and (38.5-45%) of the variables % CSL, time and % whey respectively. This could be explained by the fact that CSL and whey contain significant amounts of nitrogen, causing the increase in these effluents proportions to add directly to the nitrogen content in the medium. According to Liu & Shen (2004) and Martinez-Burgos, W. et al. (2019), hydrogen production via dark fermentation is negatively affected by nitrogen excess in the medium, which is why it is a variable that must be optimized. Another likely explanation describes that hydrogen production is also affected by COD (Sreethawong & Chatsiriwatana (2010). In this case, the optimal value reached was 31.75 g O_2 L^{-1}, since small increases in the proportions of CSL and whey result in high amounts of COD, given that both effluents have extremely high organic loads. In the case of the time variable, it can be explained by the fact that hydrogen is a metabolite associated with microbial growth. Hence, it is worth noting that only one-third of the organic

compounds available are converted in biohydrogen due to metabolic limitations since part of the substrate is directed to VFA's production, which are products related to natural survival and growth of microorganisms, being acetate directly linked to ATP formation and reduced organic acids relating to optimum redox balance due to oxidation of NADH (Hallenbeck et al., 2012).

In contrast, other hydrogen production works that utilize *C. beijerinckii* also showed that the reaction was carried out in approximately 48 h (Skonieczny & Yargeau (2009), when the highest accumulated hydrogen production is reached

FIGURE 2 - CONTOUR SURFACES OBTAINED FROM THE MEDIA OPTIMIZATION FOR CLOSTRIDIUM BEIJERINCKII



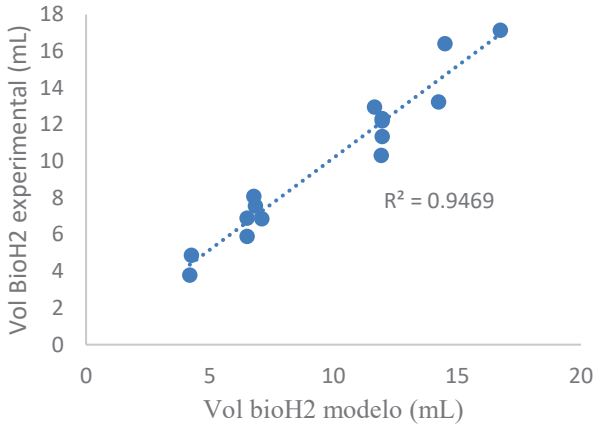
SOURCE: The author (2020).

3.3.3 Mathematical model

A regression analysis of the data allowed to obtain a mathematical model (Equation 1) with a correlation coefficient R^2 = 95 %. This demonstrates that the three variables explain the 95% of the response variable and that the remaining 5 % can be attributed to variables not controlled in the process. According to Hye et al. (2008) and Martinez-Burgos et al. (2020), models with a correlation coefficient greater than or equal to 80 % are adequate to explain the behavior of the response variable. This result can be seen in FIGURE 3, in which a coefficient R^2 = 0.946 was obtained, indicating that the modeled data follows a behavior similar to the experimental data.

$$H_2 = 11.96 + 3.7CSL + 2.58t - 1.29CSL^2 - 2.74t^2 + 2.54CSL * t$$

FIGURE 3 - ACTUAL VALUES VS PREDICTED VALUES OBTAINED USING DATA FROM THE OPTIMIZATION OF MEDIA FOR C. BEIJERINCKII



SOURCE: The author (2020)

On the other hand, it is observed that both the linear terms and the quadratic terms of the variables CSL (%) and t (h) are significant. In contrast, the terms of the whey (%) variable were not significant, which is why they were not included in the Table 2 model. The ANOVA (TABLE 3) of the model is valid because the assumptions of normality, independence and homoscedasticity are fulfilled.

TABLE 3 - CHARACTERISTICS AND PARAMETERS OF THE MODEL OBTAINED FOR CLOSTRIDIUM BEIJERINCKII

Source	Coefficient	P-value
Model		0.000
Intercep	11.96	0.000
CSL	3.70	0.000
T	2.58	0.001
W	0.13	0.71
CSL^2	-1.29	0.048
t^2	-2.74	0.003
W^2	0.34	0.526
CSL*t	2.54	0.003
CSL*W	0.96	0.100
t*W	-0.95	0.103
R^2=95.0%	Pred R^2 = 71.87%	Adj R^2=94.5%

Source: The author, 2020

3.3.4 Evaluation of bioH2 production in optimal conditions

The optimum conditions were CSL = 12 %, t = 48 h, whey = 40 %. The experimental and predicted hydrogen volumes were 18.5 ± 1.68 mL and 16.87 mL respectively. In other words, there was no significant difference between the two values ($p \leq 0.05$). FIGURE 4A shows the accumulated hydrogen production (mL). In this

figure, it is observed that in times above 48 hours there is no increase in the volume of hydrogen produced, determining that the fermentation time should not exceed 48 hours, a time when practically all common carbon sources are consumed (fructose and glucose).

It was also observed that lactic acid was consumed in parallel with these carbon sources. According to Martinez-Burgos et al. (2020) and Sikora et al. (2013), lactic acid can be used by microorganisms as an alternative carbon source for hydrogen production, however, yields are low. The fast depletion of lactic acid in media may be attributed to microorganisms of the genre Clostridia, since they are characterized for being strictly anaerobes and can assimilate lactic acid to produce butyric acid. Also, lactate may interfere on the metabolic routes benefitting butyric acid producing bacteria (Etchebehere et al., 2015).

Initial and final COD given by the mixture defined as optimum concentration in the optimization was 39.52 $gO_2.L^{-1}$ and 32.30 $gO_2.L^{-1}$, respectively. The maximum yield reached was 465 mL H_2 g / COD removed, a value much higher than that reported in other studies. For example, Krishnan et al. (2016) reached a maximum yield of 215 mL H_2 g / COD removed, Zampol et al. (2015) reported a yield of 137 mL H_2 / g COD removed, and Zampol et al. (2014) reached a yield of 61.25 mL H_2 g / COD removed. A probable explanation can be associated with both CSL and whey, which contain significant concentrations of minerals, vitamins and amino acids (Hall et al., 2003 e Hofer et al., 2018). These concentrations can alter the metabolic pathways, as the components act as enzymatic cofactors for hydrogenases, improving the production of biogas (Bao et al., 2013; Lu et al., 2013; Sharma & Melkania (2018).

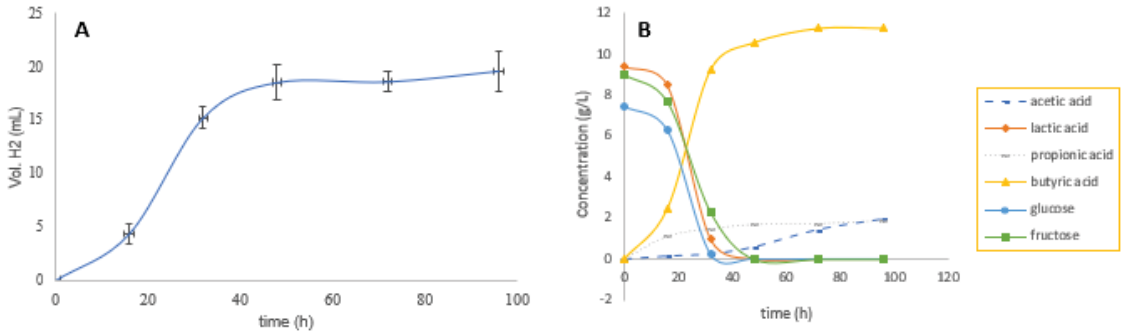
Furthermore, previous research by Pan et al. (2008b) has shown that *C. beijerinckii* is one of the microorganisms with the highest hydrogen production via dark fermentation, making it an ideal candidate for hydrogen production.

In parallel with the production of hydrogen, some intermediate metabolites are generated, mainly organic acids (FIGURE 4B), some of which are desirable while others not, precisely because of the effect they have on the production of hydrogen. Among the desirable acids are, for example, acetic and butyric acids, since for each mole of these acids 4 and 2 moles of hydrogen are produced, respectively (Sydney et al., 2020). In this case, it was observed that the butyrate route predominated, since approximately 12 g / L of this metabolite were produced. Lin, P. et al. (2007) studied the metabolic behavior of strains *Clostridium beijerinckii* and also found that the

adopted metabolic route was the butyrate with considerable productions of butyric acid compared to acetic acid.

The production of metabolites such as alcohols and propionic acid is not desirable. According to Saady (2013) for the production of stoichiometrically 1 mole of propionic acid, 1 mole of hydrogen is required, reducing its quantity during fermentation. The fact that no ethanol or other organic solvent were detected and that propionic acid concentration was low, it is possible to infer that the *Clostridium beijerinckii* strain is a promising choice for biohydrogen from soft drink wastewater, CSL and whey since alcohols and propionic are related to low yields of biohydrogen due to metabolic shifts (ZHANG & SHEN, 2007; PEIXOTO et al., 2011; WIMONSONG et al., 2014; GHIMIRE et al., 2015).

FIGURE 4 – KINETICS OF ACCUMULATED BIOHYDROGEN VOLUME (A) AND (B) KINETICS OF SUBSTRATE DEPLETION AND PRODUCT FORMATION BY CLOSTRIDIUM BEIJERINCKII



Source: The author (2020)

3.4 CONCLUSION

The four agro-industrial residues were characterized according to their composition of macro and microelements, in addition to some components present in solution, such as organic acids and alcohols. It was observed that CSL and Whey, low cost by-products, contain significant amounts of nutrients and can be used for the production of biohydrogen.

For the production process, it was observed that the variables that most influence the fermentation are the concentration of CSL and the fermentation time, mathematically represented by a model of R^2 = 95%. The maximum yield reached a value of 465 mL H_2 g COD removed^{-1}, a high value compared to other studies in the literature.

According to the profile of organic acids, the most likely route for obtaining hydrogen by *C. beijerinckii* was the one of butyrate, given the significant concentration of butyric acid throughout fermentation.

4 EVALUATION OF AMINO ACIDS KINETICS IN BIOHYDROGEN PRODUCTION FROM SOFT DRINK'S WASTEWATER AND CORN STEEP LIQUOR BY CLOSTRIDIUM BUTYRICUM

4.1 INTRODUCTION

Clostridia is covered by 64.6% of the group of mesophilic bacteria capable of forming spores and degrading carbohydrates to produce biohydrogen through dark fermentation with higher yields (Kanchanasuta & Prommeenate, 2016). Between them, *Clostridium butyricum* has not only shown high yields of biohydrogen but also demonstrated that it can degrade glucose, sucrose, lactose, xylose, glycerol and starch (Yin & Wang, 2017). This strain is a butyrate-producing bacteria which metabolizes glucose and other carbon sources to simultaneously produce biohydrogen, carbon dioxide, butyric acid and acetic acid through the Embden-Meyerhof-Parnas pathway

(Said et al., 2017). Throughout this Embden-Meyerhof-Parnas pathway, ATP, NADH and pyruvate are formed by glucose degradation. Pyruvate is converted to acetyl CoA and carbon dioxide to generate a reduced form of a ferredoxin molecule that will be further oxidized to produce biohydrogen (Kamalaskar et al., 2016). These analysis of pure cultures in dark fermentation can support future metabolic pathways studies (Yin & Wang, 2017).

Generally supplemented in anaerobic media to reduce the oxidation reduction potential (ORP), L-cysteine has been related to the enhancement of hydrogenases expression in anaerobic bacteria since hydrogenases and NAD/NADH activities are favored by low ORP media (Yang & Wang, 2018a; Zhao et al., 2012). Biohydrogen production is favored by some of L-cysteine properties once it can mediate the interaction between the substrate available and the bacteria present in medium and also through its association with proteins. It is also associated to improvement of growth of bacteria acting as a bioactive agent (Yang & Wang, 2018a).

Corn steep liquor is a waste and low-cost by-product of the corn wet-milling process and due to its origin, it is comprised of a large variety of nutrients, such as amino acids, vitamins and polypeptides that are employed in bioprocess for the production of penicillin by *Penicilium chrysogenum*. High carbon and nitrogen content and also being a source of vitamins and trace elements, this is commonly used to replace expensive nitrogen components, such as yeast extract (Hofer et al., 2018; Li et al., 2016; Liu et al., 2015). According to Hofer. et al, (2018), the multiphase system of CSL consists of a liquid phase which is miscible with water and a low solubitlity phase which is a solid phase.

Bioavailability of CSL remains undefined since few studies have been conducted to characterize both phases of this waste but since fermentation cost is of high importance for industrial applications, it is necessary to understand CSL contribution to the medium as a relative cheap nitrogen source with high nutritional potential. In this work, it is evaluated the application of CSL as a nitrogen source for biohydrogen production in dark fermentation and also as a replacement for L-cysteine due to its low-cost and high amino acids content.

4.2 MATERIAL AND METHODS

4.2.1 Inoculum Sampling

A sample from the consortium obtained from sugarcane cultivation soil (Bittencourt et al., 2014) was utilized to isolate a pure strain further identified in this work as *Clostridium butyricum* (DEBB-B348). Firstly, the consortium was reactivated in a vinasse based liquid medium where were added: (5 g / L) glucose, (1.0 g / L) $NaHCO_3$ and (0.5 g / L) cysteine-HCl at 85 ºC and 65 ºC, respectively. The sample was successively transferred to a MRS (Man, Rogosa & Sharpe) medium by pour plate and incubated in a Gas Pak jar.

Upon isolation, the strain was submitted to identification. The 16S rRNA gene sequences of the reference strains retrieved from NCBI (National Center for Biotechnology Information, MA, USA) were aligned using the online version of MAFFT program, version 7, with the option Auto (FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i). A neighbour-joining phylogenetic tree was constructed using the MEGA X 10.1 computer Kumar et al. (2018) based on the MSA file by MAFFT. The evolutionary distances were computed by Maximum Composite Likelihood Method Cavalli-Sforza; Edwards (1967) and maximum-parsimony Kluge; Farris (2011). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

The isolated strain was then inoculated into a pre optimization medium based of soft drink wastewater composed of: 3% CSL (v / v), (0.5 g / L) cysteine-HCl and (1.0 g / L) $NaHCO_3$. This medium was used for maintenance of the strain for 48 h in 37 ºC with subsequent transfer each 48 h.

Procedures according to BALCH et al. (1979) were adopted to guarantee an anaerobic environment. Oxygen was removed from the medium by boiling (100-105°C) under anoxic conditions under an argon atmosphere. Reduction of redox potential was ensured by the addition of $NaHCO_3$ (1.0 g/L) and Cysteine-HCl (0.5 g/L) at 85ºC and 65ºC, respectively. All experiments were conducted in Hungate tubes with a working volume of 6 mL. The tubes were sealed with bakelite caps and autoclavable rubber stoppers. The pH of the medium was adjusted to 7.0 using 35% NaOH solution.

Clostridium butyricum was submitted to tests in order to evaluate its ability of producing biohydrogen from a combination of cheese whey, corn steep liquor, EFB and expired sof drink (Guaraná Antarctica). Complement for media in v / v was soft drink wastewater.

4.2.2 Characterization of wastewaters

Soft drink wastewater (SDW) and expired Guaraná provided by the company Ambev (Almirante Tamandaré, Paraná, Brazil), cheese whey (CW) provided by the company Anila (Fernandes Pinheiro, Paraná, Brazil) and corn steep liquor (CSL) provided by the company Ingredion (Balsa Nova, Paraná, Brazil) were evaluated as promising substrates biohydrogen production through dark fermentation. The industrial wastewaters were stored at -20 °C for later use. Initially, these effluents were submitted a physic-chemical characterization and sugar contents were determined by High Performance Liquid Chromatography (HPLC).

Cations and anions were analyzed by ion chromatography (761 Compact IC, Metrohm AG). Metrosep C 3250/4.0 and Metrosep A Supp 5 - 250/4.0 columns were used for cations and anions, respectively. Concerning the cations, mobile phase was HNO_3 3.5 mM at a flow rate of 0.9 mL/min. As for the anions, mobile phase was Na_2CO_3 3.2 mM e $NaHCO_3$ 1.0 mM at a flow rate of 0.7 mL/min. Run times were 25 and 30 min for cations and anions, respectively.

The determination of the Chemical Oxygen Demand and the total nitrogen content (COD) were done using Standard Methods for the Examination of Water and Wastewater (1992).

4.2.3 Experimental design, optimization and statistical analyses

Six variables were evaluated, namely: concentration of EFB (-1;1) where -1 means absence and 1 means it was added in medium in a concentration of (5 g / L), the fermentation time (-1;1) where -1 corresponds to 24h and 1 corresponds to 48h of fermentation time, the presence of expired soft drink (-1;1) where -1 means absence and 1 means it was added in 20% (v / v), the percentage of inoculum (-1;1) where -1 means inoculum concentration of 10% and 1 corresponds to 20% (v / v). The concentration of CSL (-1;1) where -1 means absence and 1 means 10% (v / v) and cheese whey (-1;1) where -1 means absence and 1 corresponds to 40% (v / v) were evaluated in an attempt to determine the best source of nitrogen. Media were based of soft drink wastewater as complement. This planning was carried out with 6 factors and 12 runs, one for each selected strain. The Pareto diagram was used to determine the effects of significance in the experiments performed.

For *Clostridium butyricum*, upon significant effects determination, a central composite rotating design (DCCR) optimization planning (TABLE 4) with two factors, 5 central points and 13 runs was used to optimize the selected variables. The optimization provided an adjusted quadratic model equation shown below.

$$H2 = \beta o + \sum_{i=1}^{k} \beta i Xi + \sum_{i=1}^{k} \beta ii XiXi + \sum_{i=1} K - 1 + \sum_{j=1+i}^{k} \beta ij XiXj$$

This Equation provides the constant, linear, squared and interactive coefficients of the parameters inputted for optimization, which are β0, βi, βii and βij, respectively. The two independent variables (i=1-2) are represented by: $\mathbf{X_1}$ equals CSL concentration; $\mathbf{X_2}$ equals time. H_2 is the accumulated production of bioH_2. All statiscal analysis and surface responses were obtained in the software Minitab (15). Except for the linear terms, all non-significant terms obtained from the statiscal analysis were discarded.

TABLE 4 - CENTRAL COMPOSITE DESIGN FOR BIOHYDROGEN PRODUCTION USING CLOSTRIDIUM BUTYRICUM

Run	CSL concentration	Value (%)	Time	Value (h)
	Code X_1	X_1	Code X_2	X_2
1	1	14	-1	24
2	0	10	-1,41	19
3	-1	6	1	48
4	0	10	0	36
5	0	10	0	36
6	0	10	0	36

7	-1,41	4,34	0	36
8	1,41	15,66	0	36
9	1	14	1	48
10	-1	6	-1	24
11	0	10	0	36
12	0	10	1,41	53
13	0	10	0	36

SOURCE: The author (2020).

4.2.4 Kinetic profile of bioH_2 production and volatile fatty acids

Clostridium butyricum strain had the medium optimized with 12% of CSL. The missing media component is soft drink wastewater. The media were prepared according to the compositions presented above with the addition of NaHCO3 (1.0 g / L) and cysteine-HCl (0.5 g / L) at 85 ºC and 65 ºC, respectively. All experiments were conducted in Hungate tubes with a working volume of 6 mL. The tubes were sealed with bakelite caps and autoclavable rubber stoppers. The pH of the medium was adjusted to 7.0 using 35% NaOH solution. The inoculum rate used was 10% and culture media were incubated at 37 ºC upon inoculation.

Analysis of biohydrogen production and fatty acids was conducted periodically at 8h, 16h, 24h, 32h, 40h, 48h. A glass syringe was used to collect the produced gas which was further analyzed in a 490 Micro GC System gas chromatograph (Agilent), equipped with two columns (Molsieve 5Å and PoraPLOT U) and a thermal conductivity detector (μTCD). In the Molsieve 5Å column, the injection temperature was 110 ° C with an injection time of 20 ms, column temperature of 90 ° C and an initial pressure of 190 kPa. In the PoraPLOT U (PPU) column, the injection temperature was 110 ° C with a column temperature of 90 ° C and an initial pressure of 150 kPa. The peak retention time for biohydrogen was 0.4 min (Ms5A) and for CO2 and CH4 were 0.405 and 0.425 respectively in the PPU. The time for each run was 1.2 min. The mobile phase used was argon gas with a purity of 99.999%.

The composition of fatty acids produced and substrate depletion was analyzed using HPLC (High Performance Liquid Chromatography) at the same times where the biohydrogen samples were analyzed. Prior to injection of the samples (1mL), they were submitted to centrifugation at 6000 rpm for 10 min and microfiltration in cellulose acetate membranes (0.22 μm pore). The chromatograph used was an Agilent 1260

Infinity Quaternary LC with RI detector and Hi-Plex-H column. The detector and column temperature were 50 ° C and 60 ° C, respectively. The mobile phase used was 5mM H2SO4 at a flow rate of 0.6mL / min and the injection volume was 10 µL.

4.2.5 Evaluation of amino acids and presence/absence of cysteine in media

In order to determine if the corn steep liquor has significant and sufficient amounts of amino acids for a successful dark fermentation, a test was made in parallel with the kinetics. Test media was prepared exactly in the same way of other media prepared for this study but L-cysteine and sodium bicarbonate was not added in order to observe how the strain would behave without L-cysteine. Biohydrogen production was evaluated in the optimum time in accordance to the methodology specified in the kinetics and at the end it was evaluated if there was significative difference in the production of biohydrogen. All tests were run in triplicate.

Upon this test, the free amino acids content of corn steep liquor was analyzed by an automated SYKAM S433 amino acid analyzer (Eresing, Germany) using ninhydrin method in order to determine the amino acids profile during dark fermentation.

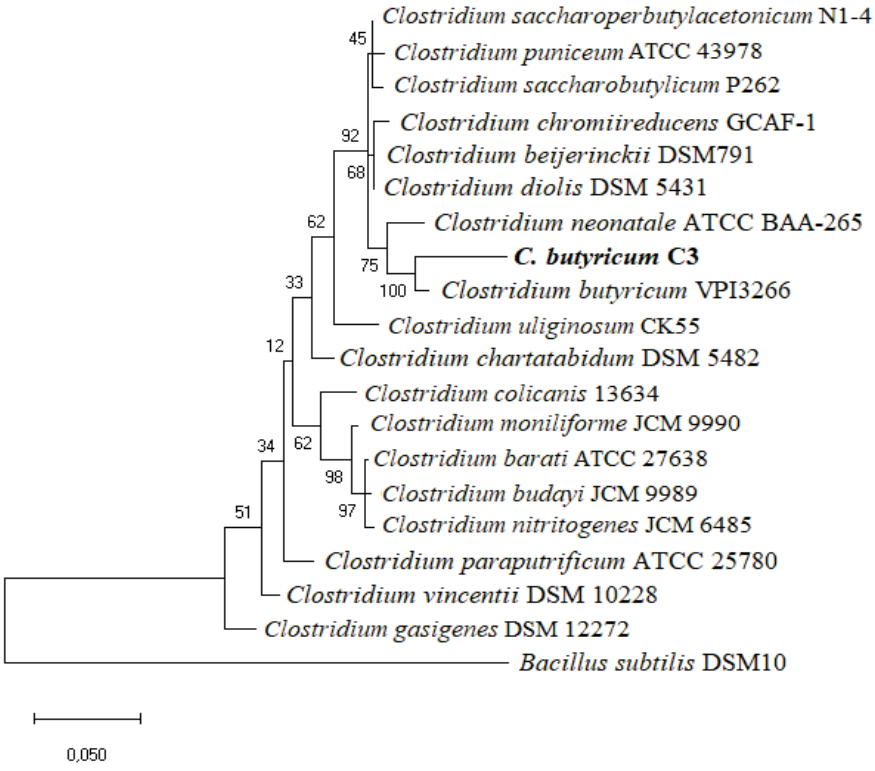
4.3 RESULTS AND DISCUSSION

4.3.1 *Clostridium butyricum* isolation

According to the BLAST analysis and phylogenetic tree (FIGURE 5), the strain isolate was identified as *Clostridium butyricum* with 99% similarity. The phylogenetic tree constructed with the neighbour- joining method revealed that the *C. butyricum* was included in the same subcluster with *C. saccharoperbutylacetonicum*, *C. puniceum*, *C. saccharobutylicum*, *C. chromiireducens*, *C. diolis*, *C. beijerinckii* and *C. neonatale*.

FIGURE 5 - MAXIMUM-LIKELIHOOD TREE BASED ON 16S RRNA GENE SEQUENCES SHOWING THE PHYLOGENETIC RELATIONSHIPS. BOOTSTRAP VALUES (%) BASED ON 1000

REPLICATIONS ARE SHOWN AT BRANCH POINTS. THE SUBSTITUTION MODEL USED WAS KIMURA 2-PARAMETER MODEL BAR, 0,05% SEQUENCE DIVERGENCE



SOURCE: The author (2020)

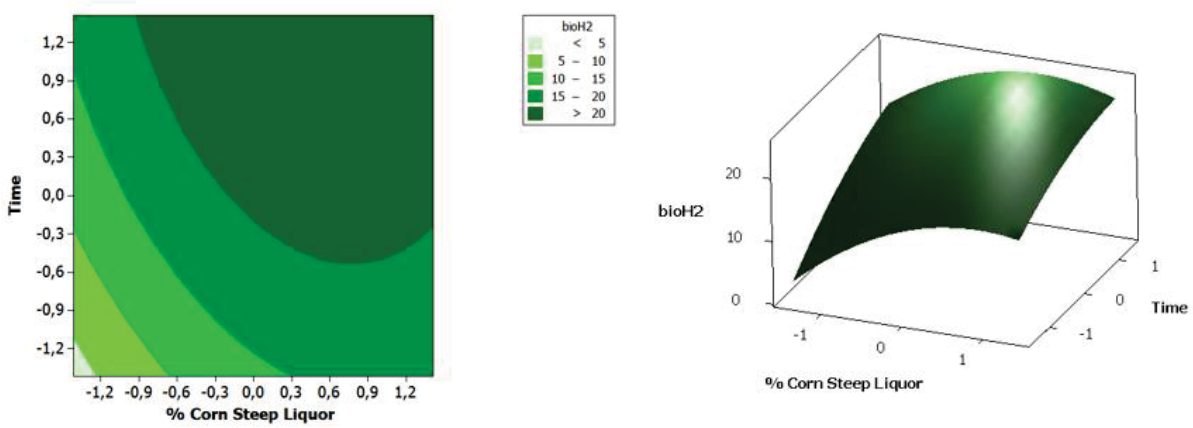
4.3.2 Variables selection and optimization

Evaluation of the residues characteristics was already done in subchapter 3.3.1. The Pareto diagram of PB (Figure not shown) showed that, of the six variables tested, only two, % v/v CSL and time, significantly affect the production of hydrogen ($p \leq 0.05$). The DCCR made possible to evaluate the optimal regions of the studied variables and criterium used for the medium component's concentration (v/v) was: minimum wastewater concentration possible to achieve maximum production in optimum conditions. Taking this into consideration, medium adopted for *Clostridium butyricum* was 12% CSL concentration, optimum time of 32h with 10% inoculum ratio. The DCCR optimization (TABLE 4) allowed obtaining the contour and surface graphics for biohydrogen production, in which the optimal regions were evaluated, as can be seen in FIGURE 6.

Same result was obtained in previous work with *Clostridium beijerinckii*. Similarities in the results may be attributed to the high organic content in corn steep liquor and its considerable amount of vitamins, metals and amino acids which are

essential for dark fermentation success once they play a vital role in cellular growth and maintenance (Hofer, et al., 2018; Yang et al., (2018). Also similar to the previous work, expired Guaraná soft drink was not significant and this may be explained considering the amount of chemical preservatives present in the final product of soft drink manufacturing. According to Kregiel (2015) and Ogueri & Vincent (2017), these chemical preservatives may cause inhibition or cellular death.

FIGURE 6 - CONTOUR AND SURFACE GRAPHICS OBTAINED WITH DCCR OPTIMIZATION FOR CLOSTRIDIUM BUTYRICUM

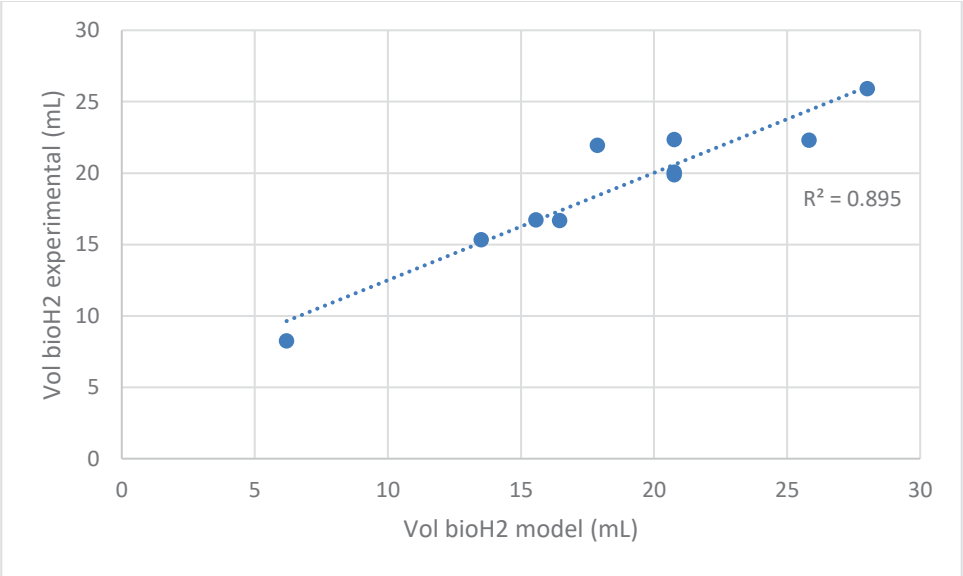


SOURCE: The author (2020).

Experimental data provided a linear regression which resulted in a polynomial model of second order. Obtained R^2 demonstrates that biohydrogen production is represented in 89.5% (FIGURE 7) of chosen intervals by the optimization equation. Martinez-Burgos et al. (2020) affirms that models with $R^2 \geq 80\%$ are satisfactory to explain the behavior of the response variable. External effects by non-controllable factors is 10.5%. The regression analysis of the data provided the mathematical model gives the equation below.

$$VH_2 = 20{,}76 + 4{,}68X_1 + 5{,}13X_2 - 4{,}76X_1^2$$

FIGURE 7 - ACTUAL VALUES vs PREDICTED VALUES OBTAINED USING DATA FROM THE OPTIMIZATION OF MEDIA FOR CLOSTRIDIUM BUTYRICUM



SOURCE: The author (2020)

4.3.3 Evaluation of kinetic profile of biohydrogen production and volatile fatty acids

The volume of biohydrogen found was 28.17 ± 0.62 mL (7.86 mmolH_2 / L.h) after 32h of fermentation with no more significant changes in biohydrogen production after this time (FIGURE 8). The yield was determined as 1067.6 mLH_2 / g $COD_{removed}$.

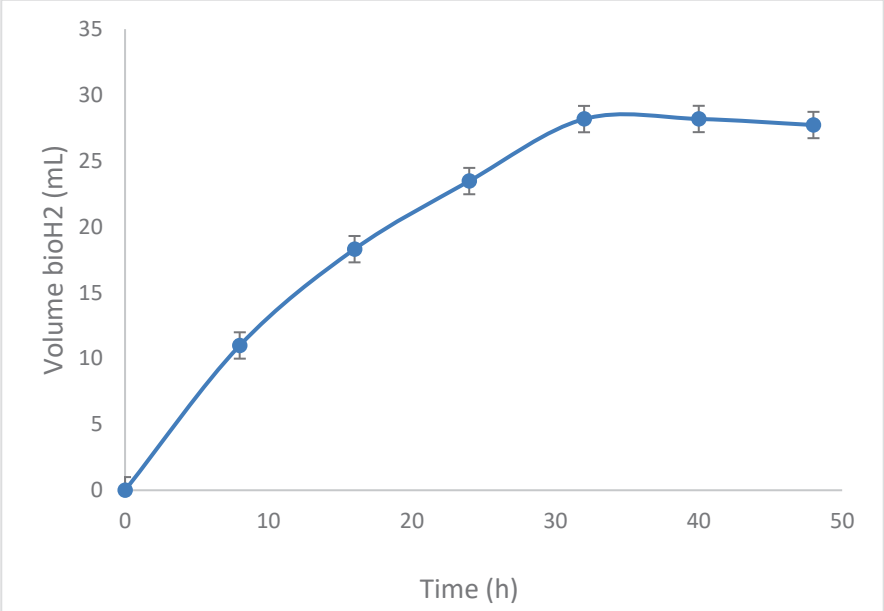
Wang et al. (2009) reached a maximum duration of 7.61 mmol / L.h using glucose (10 g / L) and *Clostridium butyricum*, however, the experiments were carried out with meat and yeast extract (high value nutrients) and agitation, which can increase not only the mass transfer in the medium, but also promote a more bioavailability for microorganisms assimilation. Stirring also increases the energy cost of the process. The study showed a short lag phase for *Clostridium butyricum* which was also found in this study (less than 8h) for the isolated strain.

Maximum cumulative biohydrogen production obtained by Yin & Wang (2017) was 218 mL / 100 mL in similar conditions with this study (pH = 7.0, 10% inoculum ratio and 35 ºC) but carbon source used was glucose with a nutrient solution. Although a nutrient solution has been employed, results are still nearly 2.5 fold lower than the obtaind by *Clostridium butyricum* in this study (28.1 mL / 5 mL).

Pattra et al. (2008) obtained 1611 mLH_2/ L.d from hydrolyzed sugarcane bagasse with 11 g/ L of glucose and 11.29 g/ L of xylose using *Clostridium butyricum* in batch and agitation. Even though this result is approximately three times higher than the obtained in this work, it is also important to note that in addition to the hydrolysis required for the release of fermentable sugars, Pattra et al. (2008) also used a nutrient

solution composed of NH_4HCO_3, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $Na_2MoO_4.2H_2O$, $CaCl_2.2H_2O$, $MnSO_4.7H_2O$ and $FeCl_2$, which makes the process more expensive. Considering a possible scaling up of the process, the use of such compounds would make hydrogen production impracticable given its high cost. Addition of supplemental solutions or hydrolysis were not necessary in this work since only low industrial waste or no added value residues were used.

FIGURE 8 - KINETICS OF ACCUMULATED BIOHYDROGEN VOLUME BY CLOSTRIDIUM BUTYRICUM



SOURCE: The author (2020)

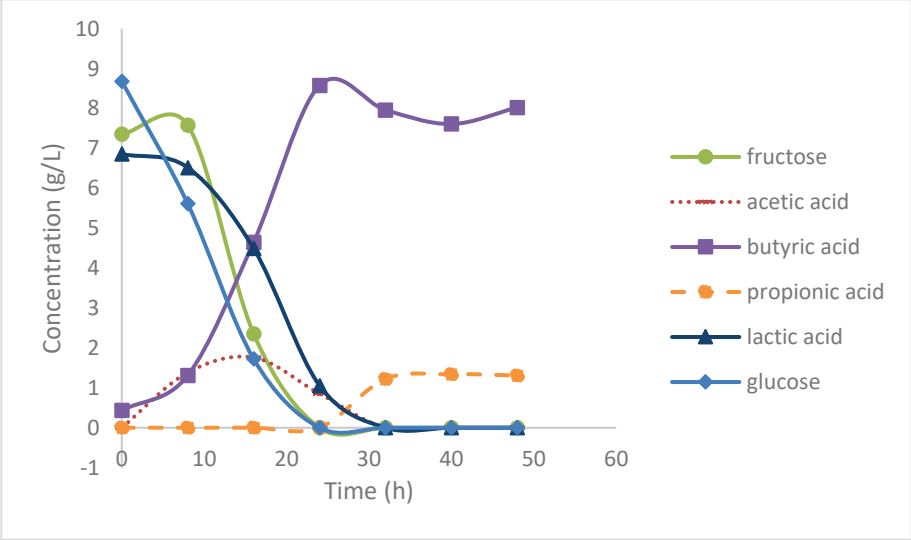
The pathway adopted by *Clostridium butyricum* was the butyrate route for energy and metabolites production. At the optimum time, the concentration of butyric acid was 7.96±0,55 g / L. Propionic acid is produced when all the glucose and lactic acid is consumed from the medium (32 h). Maximum acetic acid production is 1,76±0,03 g / L at 16 h.

Lin et al. (2007) studied the metabolic behavior of *Clostridium butyricum* strains containing and also found that the metabolic route adopted by the microorganisms was the butyrate pathway, with significant butyric acid production compared to acetic acid production. Metabolic profile of the study conducted by Kanchanasuta & Prommeenate (2016) with food waste also revealed the butyric acid fermentation was adopted by *Clostridium butyricum* once this pathway is preferable to obtain higher ATP to generate more cells. Said et al. (2017) also reported that *Clostridium butyricum* produced butyric

acid as the main volatile fatty acid followed by acetic acid reassuring that Clostridium species tend to prefer this pathway for dark fermentation. Butyric acid and acetic acid was also the main VFA's produced in the study of Seppa (2011), where it was also found a decrease in butyric acid's concentration at the end of fermentation. The relative low concentrations of propionic acid and the absence of ethanol or organic solvents indicates high biohydrogen yields once these are related to low biohydrogen yields (Ghimire et al., 2015; Peixoto et al., 2011; Wimonsong et al., 2014; Zhang&Shen, 2007).

Profile of VFA's formation is presented in FIGURE 9.

FIGURE 9 - KINETICS OF SUBSTRATE DEPLETION AND PRODUCT FORMATION BY CLOSTRIDIUM BUTYRICUM

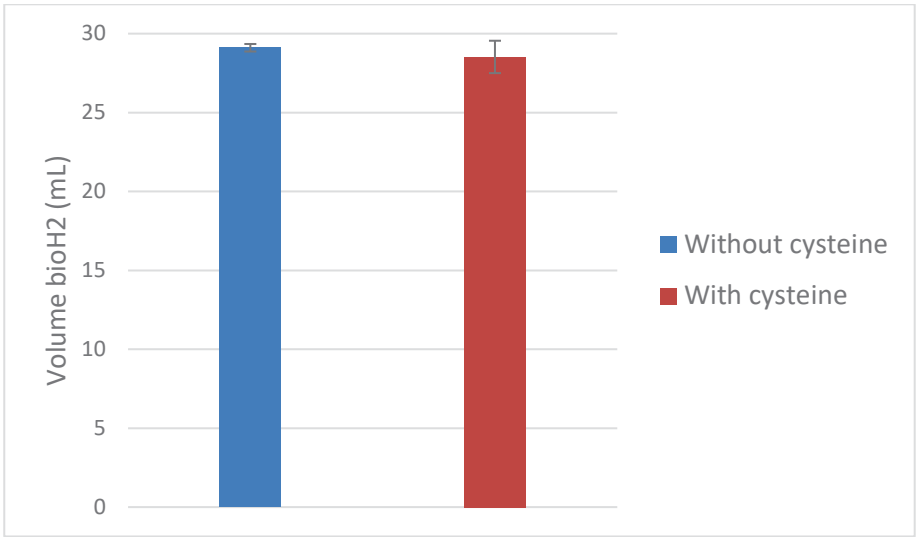


SOURCE: The author (2020)

4.3.4 Amino acids evaluation in medium

At the end of the kinetics, the volume of biohydrogen in the tubes prepared without L-cysteine and sodium bicarbonate was also measured. It is possible to verify in FIGURE 10 that no significant difference between the production of biohydrogen with and without L-cysteine and sodium bicarbonate was found. It can be inferred then that the presence of L-cysteine in the CSL fulfills the supplementation need for this amino acid in medium. It is known that the cost of the dark fermentation is a limiting factor for its application and replacing L-cysteine for CSL results in a significant decrease in production costs which could allow a promising future industrial scale up.

FIGURE 10 – COMPARISON BETWEEN BIOHYDROGEN PRODUCTION IN MEDIA WITH AND WITHOUT CYSTEINE



SOURCE: The author (2020)

Few studies were found about the characterization of corn steep liquor, especially in an amino acid level. It was assumed that CSL would contain L-cysteine in its composition once Hofer et al. (2018) had already characterized corn steep liquor in depth, observing the presence of 21 amino acids, organic acids, reducing sugars, water-soluble vitamins and other trace minerals. The author found small amounts of vitamins and trace elements in CSL, mostly associated with proteins, and it was also noticed that the main carbon source present in CSL is lactic acid but differently of this study, other sugars such as glucose and fructose were not found in expressive amounts.

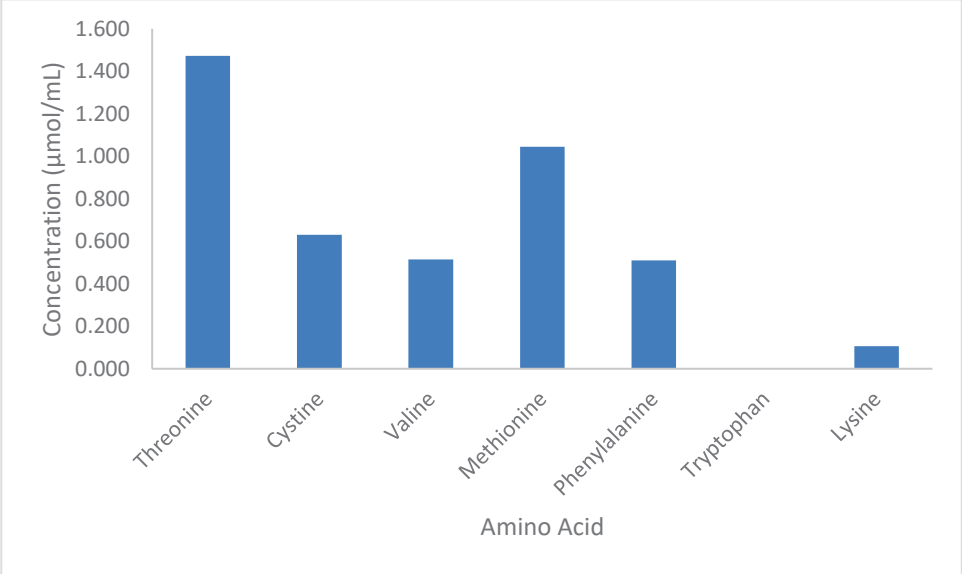
Comparatively, this work and the study of Hofer et al. (2018) may have discrepancies since in this work, it was observed the liquid phase of CSL and the other author not only characterized the liquid phase but also the solid phase of CSL by chemical and enzymatic hydrolysis. Initially, as it was stated by both authors, phenylalanine is freely available in CSL, but according to Hofer et al. (2018), less than 10% of this amino acid is still bonded to proteins. Regarding lysine in this study in comparison to Hofer's, its low concentration may be explained due to the fact that lysine is not initially available for consume or transformation, being mostly bonded to proteins or peptides. Bioavailability of cystine in this study is around 14% of total amino acids initially detected while Hofer's was 27%. These differences may not only attributed to the wider hydrolysis carried in Hofer's study but also to the variability in CSL due to different regions and corn crops. Upon chemical hydrolysis, the author

stated that amino acids with the highest solubilizations were the ones that already occurred freely in CSL, such as alanine, leucine, phenylalanine or isoleucine.

High amounts of sulfur proteins which contain methionine and cysteine may result in sulfide production which can be toxic to biohydrogen producing bacteria. It can also decrease the bioavailability of macronutrients as iron, important to hydrogenases activity, due to the formation of insoluble metal sulfides. Although this should be considered a concern in dark fermentation, concentrations of these amino acids were relatively low in CSL (Elbeshbishy et al., 2017).

All amino acids detected in the characterization of CSL were also detected by Yang et al. (2018) who used CSL to produce 2,3 butanediol. Amino acids initial concentration is shown in FIGURE 11.

FIGURE 11 – DETERMINATION OF AMINO ACID CONCENTRATION PRESENT IN THE LIQUID PHASE OF CORN STEEP LIQUOR

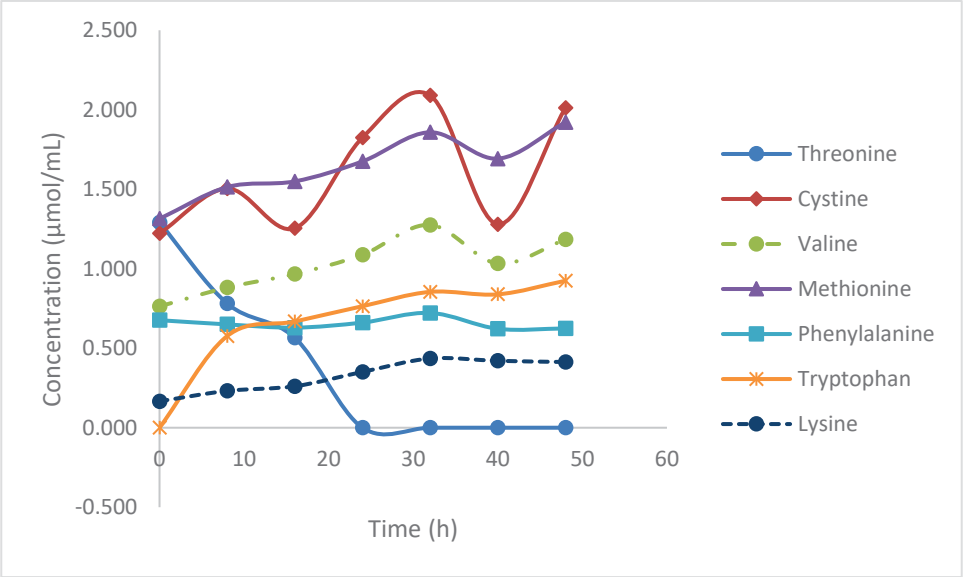


SOURCE: The author (2020)

It is important to notice in FIGURE 12 that amino acids kinetics are related to microbial enzymatic hydrolysis through proteases or peptidases. Hofer et al (2018) studied the kinetic profile of amino acids release by the fungus *P. chrysogenum*, known for penicillin production, and proved that enzymes produced by microorganisms can act hydrolytically to release amino acids bond to more complex structures. Consequently, changes in bioavailability of these amino acids may happen due to release of bound forms to a unique amino acid. According to D'Este et al. (2018), despite microorganisms can convert sugars in amino acids, amino acids like lysine,

threonine, alanine, tyrosine, histidine, etc. can also be metabolized to serve as energy source for anaerobic bacteria.

FIGURE 12 – AMINO ACID KINETICS PROFILE THROUGHOUT DARK FERMENTATION BY CLOSTRIDIUM BUTYRICUM



SOURCE: The author (2020)

Methionine and cysteine are precursors amino acids for cystine production so a strong relationship between them is expected in medium. FIGURE 12 shows the linkage between methionine and cystine, whenever cystine concentration decreases, methionine concentration is submitted to a slight increase that is followed by an increase of cystine concentration concomitantly. This may be attributed to cysteine liberation in medium.

Threonine is the most abundant amino acid initially found in CSL but it is promptly consumed before 24 h of fermentation. No threonine is replenished along with the rest of the amino acids so it may be assumed that *Clostridium butyricum* does not have the proper enzymes to set this free from complex proteins or it is no longer necessary in fermentation. However, threonine and methionine are directly bonded to acetate and butyrate production as major volatile fatty acids, especially increases in propionate concentration has been reported due to threonine supplementation in medium (Barker, 1981). As threonine is completely depleted at 24 h, simultaneously propionic acid production starts.

Lysine, valine and phenylalanine had their concentrations kept almost constant during the whole process. Tryptophan was not available initially. According to Barker

(1981), phenylalanine and tryptophan are associated to aromatic product formation such as phenylpropionic from phenylalanine acid and indole from tryptophan, and lysine can be converted to acetate and butyrate (Durre, 2005).

It is hard to determine the exact function of corn steep liquor in medium because these behaviors are tied to several factors (Yang et al., 2018). Besides, many species of Clostridia have the ability to degrade 12 from 20 proteinogenous amino acids (Durre, 2005), therefore, a complex analysis is further necessary to state a clear relation between amino acids kinetics and dark fermentation.

4.4 CONCLUSION

Clostridium butyricum achieved high biohydrogen yields from low-cost residues such as corn steep liquor and soft drink wastewater. Yields related to this pure strain was higher than most of the studies that used *Clostridium butyricum* and may be compared to yields attributed to studies conducted with consortia. It was proved that CSL may be a low-cost replacement for L-cysteine in medium by amino acid analysis of this dual-phase residue. Deeper analysis must be done in CSL to characterize this residue not only in liquid phase but also through its solid phase. It was determined a strong linkage between dark fermentation and amino-acids consumption. The consumption of threonine is strongly bonded to propionic acid formation. It is concluded that CSL composition has the necessary amino acids to carry and improve dark fermentation. The major conclusion is that CSL is an alternative for amino acids in dark fermentation media, especially when related to replacing expensive purified substrates as cysteine in media.

Amino acids kinetics throughout fermentation was conducted and due to its complex metabolism and structure, more analysis must be done to come to a conclusive determination of their influence in dark fermentation.

5 ENHANCEMENT OF BIOHYDROGEN PRODUCTION IN INDUSTRIAL WASTEWATERS WITH VINASSE POND CONSORTIUM USING GREEN IRON NANOPARTICLES

5.1 INTRODUCTION

The increasing emission of greenhouse gases, depletion and possible scarcity of fossil fuels in the future have led to search for alternative fuels. Hydrogen has been considered “the fuel of the future” as it presents a high energy content of 120 MJ/kg, which is 346% and 175% higher than that of ethanol and gasoline, respectively (Li et al., 2020). Currently, approximately 48% of the hydrogen produced comes from natural gas, 30% from heavy oils and naphtha, 18% from coal, and 3-4% from electrolysis of water and only 1% from biological origin (Abubackar et al., 2019; Li et al., 2020).

Biological methods, developed at ambient temperatures and pressures, mainly include photosynthetic and dark fermentative hydrogen production (Cisneros de la Cueva et al., 2018). Dark fermentation is the preferred technology for its higher production rate and less energy requirement. The process can also be driven under non-sterile conditions using either pure culture or mixed consortium (Sivagurunathan & Lin, 2020).

Thus, in order to obtain a sustainable and cost-effective process of biohydrogen production, many wastes have been employed, including brewery wastewater (Arantes

et al., 2020), cassava wastewater (Mari et al., 2020), agricultural residues or wastewater from food industries (Mahato et al., 2020) and even organic fractions of municipal solid wastes (Shah et al., 2016).

Nanotechnology in biohydrogen production has made use of iron nanoparticles promoting the activity of key enzymes, accelerating the electron transfer between ferredoxin and hydrogenase (Yang & Wang, 2018). Some works present increases of 34% and 5% in the hydrogen (Engliman et al., 2017).

However, the classic methods for nanoparticle synthesis makes use of toxic and non-biodegradable compounds. Thus, the use of green synthesized nanoparticles on biohydrogen production must be also encouraged.

The aim of this work is to produce biohydrogen using industrial wastes as carbon source. The production yields were also compared with the addition of green synthesized iron nanoparticles using lignin extracted from oil palm empty fruit bunches as capping agent.

5.2 MATERIAL AND METHODS

5.2.1 Inoculum sampling

The anaerobic environment was created following the technique described by Balch et al. (1979). The removal of the oxygen from the medium was made by boiling (100-105°C) the medium under anoxic conditions (argon atmosphere). The redox potential of the medium was reduced adding $NaHCO_3$ (1.0 g/L) and Cysteine-HCl (0.5 g/L) at 85ºC and 65ºC, respectively. All cultures were done on 16 mL Hungate type tubes with a working volume of 6 mL. The tubes were sealed with bakelite caps and autoclavable rubber stoppers. The pH of the cultures was initially adjusted to 7.0 using 1.0 M NaOH.

A consortium used by Bittencourt et al. (2014), and later identified by (citar Drielly), LPB AH8 (vinasse pond), was tested in order to produce biohydrogen. This consortium is composed of 34.5% *Ruminococcus*, 64% *Clostridium* and 1.5% other genera. The strains were reactivated in a liquid medium based on vinasse with the addition of glucose (5 g / L) and the addition of $NaHCO_3$ (1.0 g / L) and cysteine-HCl (0.5 g / L) at 85 ºC and 65 ºC, respectively. After reactivation, consortium was then inoculated in a medium containing washing effluent from soft drink production, 3% CSL (v / v), cysteine-HCl (0.5 g / L) and $NaHCO_3$ (1.0 g / L). The maintenance and

conservation of the consortia were carried out by weekly transfer to fresh culture media.

5.2.2 Characterization of residues

Soft Drink's wastewater (SDW) and expired Guaraná Antarctica soft drink were provided by Ambev Company (Almirante Tamandaré, Paraná, Brazil). Corn steep liquor (CSL) was obtained by Ingredion Company (Balsa Nova, Paraná, Brazil). These liquid effluents were used as substrates for biohydrogen production. The residues were stored at 20 °C for later use. Sugar content of the effluents were analyzed by High Performance Liquid Chromatography (HPLC). Samples (1.5 mL) were centrifuged at 6000 rpm for 10 min and submitted to microfiltration on cellulose acetate membranes (0.22 mm). Analysis were run in a chromatograph Agilent 1260 Infinity Quaternary LC with RI detector and Hi-Plex-H column. Detector and column temperatures were 50 ºC and 60 ºC, respectively. The mobile phase was 5 mM H2SO4 at a flow rate of 0.6 mL/min and the injection volume was 10 mL.

Cations and anions were analyzed by ion chromatography (761 Compact IC, Metrohm AG). Metrosep C 3250/4.0 and Metrosep A Supp 5 - 250/4.0 columns were used for cations and anions, respectively. For cations, the mobile phase was 3.5 mM HNO_3 at a flow rate of 0.9 mL/min. For anions, the mobile phase used was 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$ at a flow rate of 0.7 mL/min. Run times were 25 and 30 min for cations and anions, respectively.

Total nitrogen (Kjeldahl) and chemical oxygen demand (COD) were done using Standard Methods for the Examination of Water and Wastewater (1992). Characterization data can be seen in TABLE 5.

TABLE 5 - PHYSICAL-CHEMICAL COMPOSITION OF SOFT DRINK WASTEWATER AND CORNT STEEP LIQUOR.

Parameters	CSL	Soft Drink Wastewater
K (mg L^{-1})	38,000	38.3
Fe (mg L^{-1})	<100	3.0
Mn (mg L^{-1})	60.0	-
Cu (mg L^{-1})	47.0	-
Zn (mg L^{-1})	60.0	-
Na (mg L^{-1})	1,412	268.6
Ca (mg L^{-1})	1,170	43.0
Mg (mg L^{-1})	2,875	32.45
NH_4 (mg L^{-1})	1,412	5.85
NO_3 (mg L^{-1})	-	16.4

PO_4 (mg L^{-1})	-	-
F (mg L^{-1})	25.057	-
Cl (mg L^{-1})	36.576	87.8
Citric Acid (g L^{-1})	-	1.182
Fructose (g L^{-1})	35.49	3.998
Lactic Acid (g L^{-1})	92.55	0.5676
Acetic Acid (g L^{-1})	0.3617	0.3071
Maltose (g L^{-1})	1.493	-
Glucose (g L^{-1})	36.31	0.7
Lactose (g L^{-1})	-	-
Nitrogen (g L^{-1})	10.8*	0.5
Phosphorus P_2O_5 (g L^{-1})	<1.0*	<1.0
pH	4.32	7.56
COD (g L^{-1})	245.4	4.27

*Concentration given in $mg.g^{-1}$
SOURCE: The author (2020)

Expired Guaraná Antarctica's composition was considered as the same found in the label of the bottle defined by carbohydrates (20 g) and sodium (11 g) at each portion of 200 mL, critic acid, sodium benzoate and potassium sorbate.

5.2.3 Experimental design, optimization and statistical analyses

The influence of 6 different factors on biohydrogen production was evaluated. The factors evaluated were EFB presence/absence, Guaraná soft drink presence/absence, inoculum concentration, fermentation time and CSL concentration. The experiment design consisted on 12 runs for each selected strain. Pareto charts (data not shown) were used to graphically determine if each factor exhibited significant influence on the dependable variable. After selecting the most influencing variables, a central composite design (DCCR) was ran in order to optimize the biohydrogen production according to TABLE 6. Experiments were run using LPB AH8 consortium.

The optimizations were adjusted to a quadratic model presented in Equation 1.

$$H2 = \beta o + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta iiXiXi + \sum_{i=1} K - 1 + \sum_{j=1+i}^{k} \beta ijXiXj \qquad \textbf{Equation 1}$$

Where β0, βi, βii and βij are the constant, linear, squared and interactive coefficients of the input parameters, respectively. Where (i = 1-2), are the two independent variables ($\mathbf{X_1}$: CSL concentration; $\mathbf{X_2}$: time), and H_2 is the accumulated production of bioH_2. All statistical analysis and surface responses were performed

using the software Minitab (15). The non-significant terms (interactions and quadratics) of the models were excluded, except for the linear terms.

TABLE 6 - CENTRAL COMPOSITE DESIGN FOR BIOHYDROGEN PRODUCTION SUING LPB AH8 CONSORTIUM

Run	CSL concentration	Value (%)	Time	Value (h)
	Code X_1	X_1	Code X_2	X_2
1	1	14	-1	24
2	0	10	-1,41	19
3	-1	6	1	48
4	0	10	0	36
5	0	10	0	36
6	0	10	0	36
7	-1,41	4,34	0	36
8	1,41	15,66	0	36
9	1	14	1	48
10	-1	6	-1	24
11	0	10	0	36
12	0	10	1,41	53
13	0	10	0	36

SOURCE: The author (2020)

5.2.4 Kinetic profile of biohydrogen production and volatile fatty acids

With the results of the optimizations, the medium adopted for LPB AH8 consortium was defined as 12% of CSL in optimum time equal to 48 h. The missing media component is residual water from soft drink production. The media were prepared according to the compositions presented above with the addition of $NaHCO_3$ (1.0 g / L) and cysteine-HCl (0.5 g / L) at 85 ºC and 65 ºC, respectively. All fermentations were carried out in 16 mL Hungate tubes with a useful volume of 6 mL. The tubes were closed with bakelite caps and autoclavable rubber caps. The pH of the media was adjusted to 7.0 using 35% NaOH (w/v). The inoculum rate used was 10%. Hungate tubes were incubated at 37 ºC after inoculation.

The production of biohydrogen and fatty acids was analyzed periodically at 16h, 32h, 48h, 72h and 96h. The gas produced was collected using glass syringes (10 mL

and 100 mL) and analyzed in a 490 Micro GC System gas chromatograph (Agilent), equipped with two chromatographic columns (Molsieve 5Å and PoraPLOT U) and thermal conductivity detector (μTCD). In the Molsieve 5Å column, the injection temperature was 110°C with an injection time of 20 ms, column temperature of 90°C and an initial pressure of 190 kPa. In the PoraPLOT U (PPU) column, the injection temperature was 110°C with a column temperature of 90°C and an initial pressure of 150 kPa. The peak retention time for biohydrogen was 0.4 min (Ms5A) and for CO_2 and CH_4 were 0.405 and 0.425 respectively in the PPU. The time for each run was 1.2 min. The mobile phase used was argon gas with a purity of 99.999%.

The organic compounds produced during fermentation were also analyzed using a high performance liquid chromatograph (Agilent 1260 Infinity Quaternary LC) equipped with RI detector and Hi-Plex-H column. The detector and column temperature were 50°C and 60°C, respectively. The mobile phase used was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min and an injection volume of 10 μL. 1 mL samples of fermentation media were centrifuged (6000 rpm) for 10 min and filtrated using cellulose acetate membranes (0.22 μm pore).

5.2.5 Lignin extraction from oil palm empty fruit bunches

Oil palm empty fruit bunches (OPEFB) were obtained from Biopalm Vale factory, located in Mojú (Pará), Brazil. OPEFB were dried in an air-circulating oven at 65°C for 48 h, milled in a knife mill (Marconi, MA580/E) and the particle size used was between ASTM No. 20 (0.85 mm) and ASTM No. 45 sieves (0.35 mm). Lignin was extracted applying a sequential acid-alkaline pretreatment. First, the material was submitted to a dilute acid hydrolysis under the following conditions: 10 g of dry EFB / 100 g of total mass, 2.9 g of sulfuric acid / 100 g of total mass at 125°C for 25 minutes. The resulting products of the acid hydrolysis were filtered and the solid fraction was washed twice with deionized water. The solid fraction was dried at 45°C for 24 h and further submitted to the alkaline extraction process in an autoclave at 121°C for 60 minutes with 2.5% wt. and 10 wt.% of NaOH and dried solids from the acid hydrolysis process, respectively. The liquid fraction containing the extracted lignin was acidified with 72% wt. H_2SO_4 until pH 2.0 and then filtered. The precipitate was washed three times with acid water followed by three washes with hot water. Finally, the washed lignin was dried at 80°C for 8 h. For all the experiments, lignin was solubilized in alkaline water (pH 11) with agitation. Then, the solution pH was adjusted to 6-7 using 2% wt. H_2SO_4.

5.2.6 Green iron nanoparticles synthesis

Lignin magnetic iron nanoparticles (LMNPs) were prepared by precipitation method Abbasi Kajani; Bordbar (2019). Stock solutions of 0.2 M $FeCl_3.6H_2O$ and 0.2 M $FeSO_4.7H_2O$ were prepared separately by dissolving the iron salts in distilled water. Two volumes of Fe^{3+} solution were mixed with one volume of Fe^{2+} solution and five volumes of 1.6 mg/mL lignin solution. The solutions were mixed and stirred, while slowly adding 2mL of 2 M NaOH. The reaction proceeded for 30 min at 60°C in water bath, and the resulting LMNPs were purified repeatedly by centrifugation with excess ultrapure water until a neutral pH level was reached.

Lignin non-magnetic iron nanoparticles (LNMNPs) were prepared by mixing 2 volumes of 0.2 M $FeSO_4.7H_2O$ with five volumes of 1.6 mg/mL lignin solution and three volumes of water. The reaction proceeded for 5 h at 60°C in water bath, and the resulting LNNPs were purified repeatedly by centrifugation with excess ultrapure water until a neutral pH level was reached (Fazlzadeh et al., 2017).

5.2.7 Nanoparticles characterization

The morphology and size were determined by Transmission Electron Microscopy (TEM) (JEOL JEM-1200EX-II) operating at 120 kV. The elemental composition of nanoparticles was obtained using Energy Dispersive X-Ray (EDS). The crystal structure information was obtained using XRD-7000 (Shimadzu) X-ray diffractometer with Cu Kα radiation (λ = 1,7890 Å) operating at 40 KV and 20 mA. FTIR measurements of lignin and iron nanoparticles (FeNPs) were made in the wave range of 400-4000 cm^{-1} using a Vertex 70 (Bruker) spectrometer.

5.2.8 Addition of nanoparticles in media

To evaluate the influence of nanoparticles in media, experiments were run varying the concentration of the two nanoparticles in optimized media. Concentrations tested were 50 mg / L, 100 mg / L, 200 mg / L, 300 mg / L and 400 mg / L. Biohydrogen and volatile fatty acids determination were studies in optimum time defined by the optimization in order to compare the productions between media with nanoparticles and media without nanoparticles.

5.3 RESULTS AND DISCUSSION

5.3.1 Iron nanoparticles characterization

The size and morphology of iron nanoparticles is shown in FIGURE 13. LMNP exhibited almost spherical shape with an average size of 8.6 nm with low agglomeration (FIGURE 13a). On the other hand, LNMNP presented high agglomeration between the particles, not being possible to measure the particle average size (FIGURE 13b).

The weight percentage of iron, oxygen and carbon present in the nanoparticles from EDS study is shown in FIGURE 13 for both nanoparticles. It was observed that LMNP and LNMNP presented a carbon content of 6.5 and 40.9%, respectively. The analysis confirmed the capping action of lignin in both nanoparticles.

FTIR spectra for lignin, LMNP and LNMNP is found in FIGURE 14a. The following structures were found in lignin: hydroxyl groups (O-H stretching) in aliphatic and aromatic structures at 3300-3400 cm^{-1} (Aadil et al., 2016), C-H stretching at 2931 and 2850 cm^{-1} (Isroi et al., 2012), ester linkage of carboxylic group of ferulic and p-coumaric group acids at 1709 cm^{-1} Flauzino Neto et al. (2013), C=C stretching of the aromatic ring (S) at 1598 cm^{-1} (Carrillo et al., 2018), C=C stretching of the aromatic rings at 1511 and 1465 cm^{-1} (Carrillo et al., 2018; Kim et al., 2013), vibrations with C-H in-plane deformation CH_2 scissoring at 1424 cm^{-1} Isroi et al. (2012), O-H from secondary alcohols at 1324 cm^{-1} Constant et al. (2015), ether bridges at 1226 cm^{-1} Alriols et al. (2010), aromatic C-H in plane deformation at 1121 cm^{-1} (Singh & Dhepe, 2016), C-H in aromatics at 1031 cm^{-1} (Constant et al., 2015). The signals exhibited at 911 and 836 cm^{-1} corresponded to =CH out-of-plane deformation and aromatic C-H out-of-plane deformation, respectively (Toledano et al., 2010). It was noticed that both iron nanoparticles exhibited the same functional groups found in lignin, meaning theat lignin is acting as capping agent. Additionally, LMNP and LNMNP exhibited bands between 400 and 600 cm^{-1}, which are related to Fe-O stretching vibrations, indicating the formation of iron nanoparticles (Bishnoi et al., 2017; Karpagavinayagam & Vedhi, 2019). However the intensity of the peaks is small compared to those from lignin as the latter acts as coating agent (Singh et al., 2017).

The XRD patterns of the iron nanoparticles are presented in FIGURE 14b. The LMNP showed peaks at 2θ values of 35.6°, 43.6°, 57.3°, and 63°, which can be

indexed as reflection planes of (311), (400), (511), and (440), respectively for magnetite (Fe_3O_4). The XRD pattern for LNMNP showed that the particles were amorphous.

FIGURE 13 - TEM AND EDS ANALYSES OF A) LMNP B) LNMNP

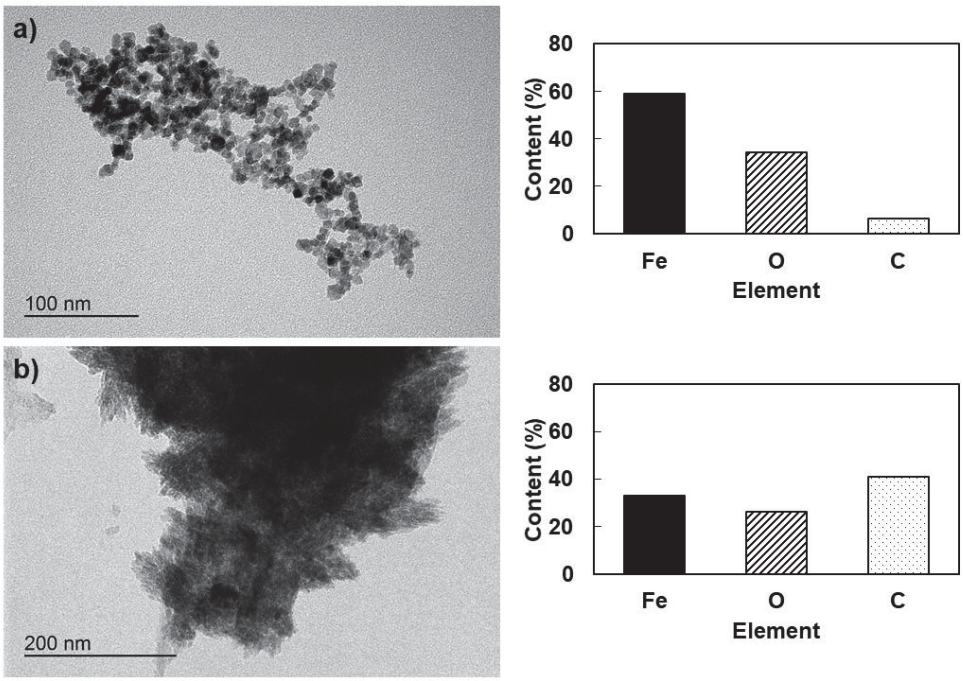
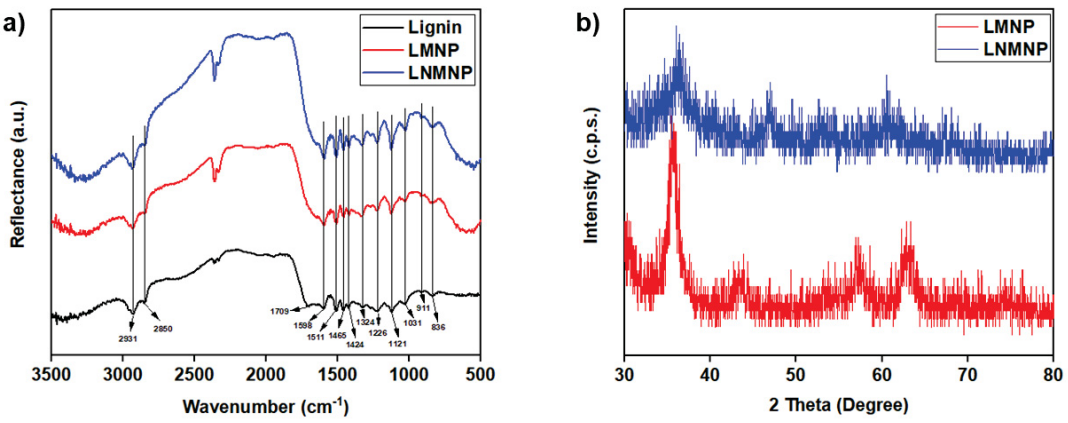


FIGURE 14 - A) FTIR AND B) XRD ANALYSES OF LMNP AND LNMNP



SOURCE: The author (2020)

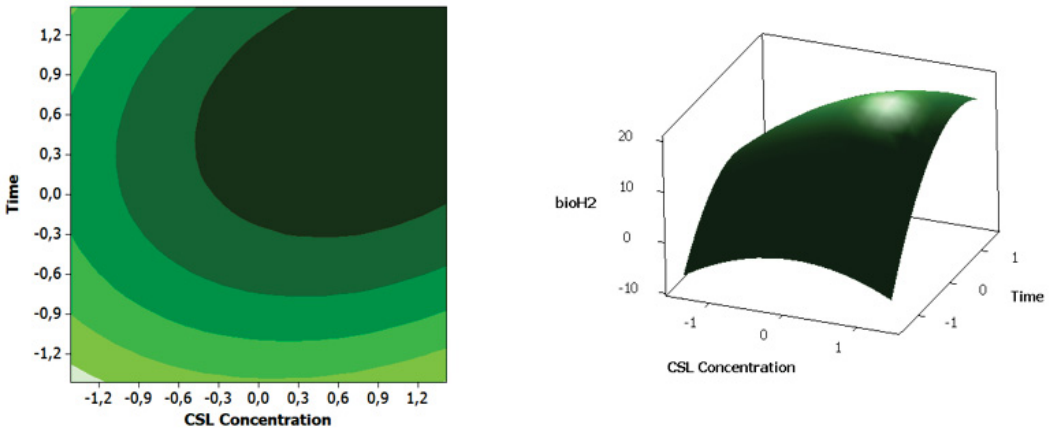
5.3.2 Optimization

Only corn steep liquor and time of fermentation were significant considering the Pareto graphic. Evaluating the production of biohydrogen by *Clostridium beijerinckii*

with expired soft drink and soft drink wastewater in Chapter 3 of this work, it was found that expired soft drink (Guaraná) wasn't significant for biohydrogen production. This is due to its antimicrobial properties in the finished product, characterized by the presence of chemical preservatives as sorbates and benzoates, responsible for causing inhibition of amino acids uptake and destroys the internal proton level of microbial cells (Kregiel, 2015).

The DCCR optimization (TABLE 6) allowed obtaining the contour and surface graphics for LPB AH8 consortium, in which the optimal regions were evaluated, as can be seen in FIGURE 15.

FIGURE 15 – CONTOUR AND SURFACE GRAPHICS OBTAINED WITH DCCR OPTIMIZATION FOR LPB AH8



SOURCE: The author (2020)

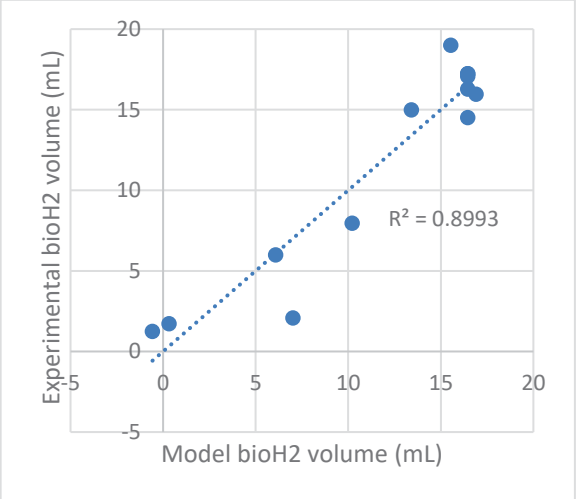
With this, it was possible to evaluate the optimal regions of the studied variables. With the results of the optimizations, criteria used for the medium components concentration (v/v) was: minimum wastewater concentration possible to achieve maximum production in optimum conditions. Following these criteria, medium composition adopted for LPB AH8 was defined as 12% of CSL in a basis of residual water from soft drink production (88%) in optimum time equal to 48h. A good justification for the fact that CSL was significant in the tests performed is the large amount of sugars, vitamins and amino acids present in its composition. In its composition are also found several metals such as iron, manganese, copper, zinc, among others, which can alter the metabolism of microorganisms to achieve greater productivity (Hofer et al., 2018; Li et al., 2016 & Yang et al., 2018).

Experimental data provided a linear regression which resulted in a polynomial model of second order. Obtained R^2 demonstrates that biohydrogen production is represented in 92% of chosen intervals by the optimization equation. External effects by non-controllable factors is 8%

$$VH_2 = 16,47 + 3,35X_1 + 4,94X_2 - 2,83X_1^2 - 5,02X_2^2$$

The equation presented does not consider non-significant terms ($p \geq 0.05$). For the LBP AH8 consortium, the linear terms and their quadratic terms were significant ($p \leq 0.05$), however, no interaction between variables represents the biohydrogen production model. The experimental values and the values predicted by the model is correlated with R^2 of 89.9% LBP AH8. As shown in FIGURE 16, the good fit of the model can be represented by the chosen variables, from which a suitable regression model for the production of biohydrogen can be generated.

FIGURE 16 – REGRESSION MODEL FOR THE PRODUCTION OF BIOHYDROGEN BY LBP AH8



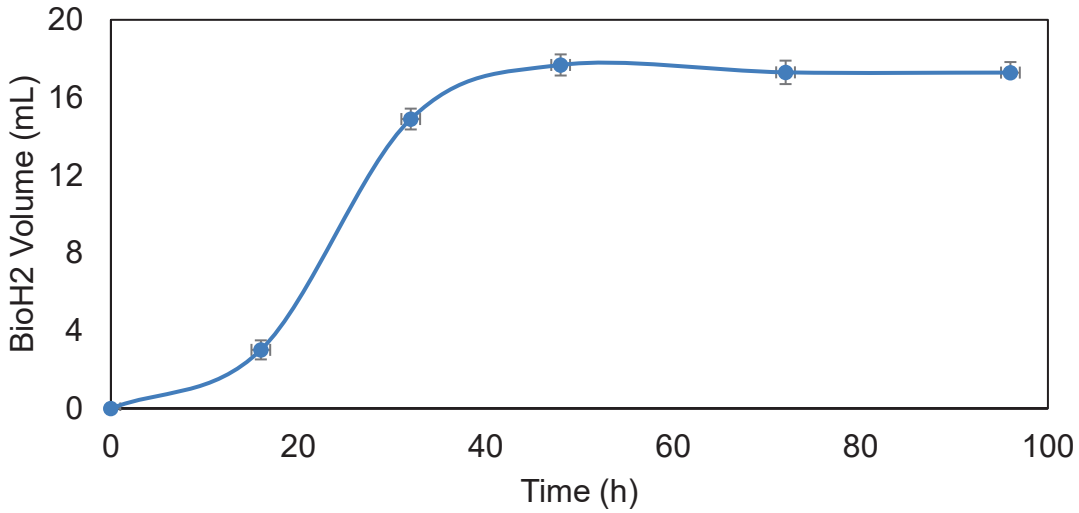
SOURCE: The author (2020)

5.3.3 Kinetic profile of biohydrogen production and volatile fatty acids

As stated in many studies about biohydrogen production, the actual yields related to its production are significantly lower than the yields predicted theoretically (Wimonsong et al., 2014). The LPB AH8 consortium obtained biohydrogen production reaching its maximum in 48 hours with a total accumulated volume of 17.67 ± 0.54 mL, with no further surplus biohydrogen production after this time. FIGURE 17 shows that

the established time is in accordance with that established by the optimization and also that, in an economic analysis, the process must be stopped at that moment to avoid death time.

FIGURE 17 – ACCUMULATED BIOHYDROGEN VOLUME PRODUCED IN KINETICS BY LPB AH8



SOURCE: The author (2020)

There are many factors that may have influenced biohydrogen production by the consortia in the dark fermentation enhancing or reducing its bioactivity. The complexity of the substrate degradation must be taken in consideration since wastewaters and organic wastes not only have naturally different compositions but they also can induce shifts in metabolic routes when decomposed, especially when consortia are used. Therefore, it is of huge importance to understand how the consortia work in order to improve biohydrogen production (Hung et al., 2011; Martinez-Burgos et al., 2019).

The consortium is composed of 34.5% *Ruminococcaceae* and 64% *Clostridiaceae*. Sydney et al. (2018) have identified *Clostridiaceae* composing consortia on his studies and it is widely known that *Clostridium* species generally are associated to high yields of biohydrogen. According to Martinez-Burgos et al. (2020), *Ruminococcaceae* is, morphologically, a diverse family comprised of strictly anaerobes with species able to produce biohydrogen, such as *Ruminococcus flavefaciens* and *R.*

albus, the last oxidizing glucose to produce biohydrogen, acetic acid, organic solvents through the Embden-Meyerhof-Parnas route.

It is possible to imply that the presence of ions in the wastewater, such as manganese, magnesium and phosphorus are important for the production of biohydrogen. Iron has a fundamental role in the activation of hydrogenases, therefore, it is indispensable for the composition of the medium (Taherdanak et al., 2015). The optimal concentration of iron in media may vary depending on the microorganisms used. Bittencourt et al. (2014) determined that the optimum concentration of iron is approximately 10 mg / L for *Clostridium pasteurianum*. As for corn steep liquor, in the characterization of Hofer et al. (2018), it was also found that the largest source of carbon, also being the main component of millet, is lactic acid (Table 1). In addition to lactic acid, 54 other components were also characterized, 21 amino acids, 5 organic acids, 8 reducing sugars, 7 vitamins and 14 trace elements. It is worth mentioning that the analyzed corn steep liquor in this work has different characteristics since the composition is dependent on the harvest and characteristics of the corn (YANG et al., 2018). Given its high content of nitrogen, vitamins and proteins, corn steep liquor can have a major impact on fermentations, since nitrogen is necessary for cell replication, maintenance of metabolism and also for the production of glycolytic enzymes (Hamilton et al., 2017).

The pH directly affects dark fermentation and can alter metabolic routes and functions, including the activation of hydrogenases and how the dynamics between microorganisms in a consortium work due to the penetration of acidic or alcoholic compounds through the cell wall. This penetration happens due to the accumulation of organic acids in the dark fermentation, inhibiting the production of biohydrogen by anaerobic bacteria since there is a strong influence of the ionic strength of non-dissociated acids in the medium. When this ionic gradient is very large, the dissociation of these VFA's inside the cells causes the deactivation of hydrogenases and the activation of enzymes that induce the production of these organic solvents. This gradient is also related to an expenditure of cellular energy since pumping protons out of cells requires the use of ATP that could be used to consume more substrate available in the medium (Elbeshbishy et al., 2017; Sivagurunathan&Lin (2019)

As pH in optimum time of the dark fermentation process was 5.2, near to the value of 5.4 found by Sivagurunathan et al. (2015), it is possible to conclude that VFA’s produced may have an inhibitory effect on biohydrogen but they are probably not the

major inhibitor in the process since most of the dark fermentations start at pH 5.5, being this pH considered satisfatory by many authors (Wu et al., 2017).

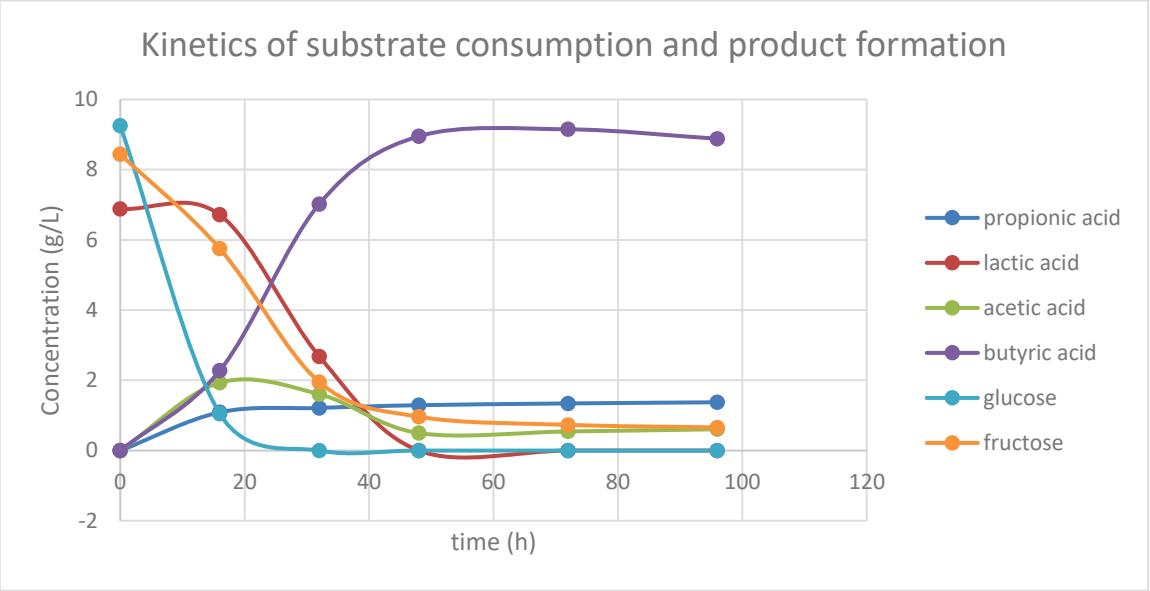
Production by the LPB AH8 consortium followed the butyrate route, with an average production of 8.95 ± 0.256 g / L of butyric acid in the optimum time (48h), as can be seen in FIGURE 18. This indicates that a favorable biohydrogen process was adopted (Chu et al., 2013; Liu et al., 2015). Bittencourt et al. (2014), also found that this consortium produced high amounts of butyric acid (10 g / L) in tests performed with vinasse supplemented with sucrose, juice and molasses. Propionic acid and acetic acid are also produced (1.29 ± 0.01 g / L and 0.498 ± 0.018 g / L), however, acetic acid is consumed when sources such as lactic acid and fructose begin to reach low concentrations and there is, practically no more glucose available making acetic acid final concentration in the medium lower.

Family Ruminococcaceae was submitted to a kinetics study by Martinez-Burgos et al. (2020) with a consortium which also was comprised with family Clotridiciaceae. This interaction resulted in a relative abundance of Ruminococcaceae between 24 and 48h of dark fermentation, period of time when most of hydrogen was already produced and the consumption of organic acids may be related to the thrive of this family in this interval too.

Several other authors also found butyric acid, acetic acid and propionic acid to be the major VFA’s produced in dark fermentation and according to Etchebehere et al. (2015), the formation of acetic and propionic acids gives an evidence that lactic acid was also consumed by other species present in the consortia. Low concentrations of propionic acid and no ethanol or organic solvents identification are promising results since the presence of the compounds are related to low biohydrogen yields (Ghimire et al., 2015; Peixoto et al., 2011; Wimonsong et al., 2014; Zhang&Shen, 2007).

During fermentation, lactic acid and fructose start to be effectively consumed when the glucose available in the medium is practically depleted around 20 h of fermentation. At the end of fermentation, it is possible to find a trace amount of fructose, practically the same concentration of acetic acid. After reaching its maximum concentration at 48h, butyric acid starts to be slightly consumed decreasing minimally its concentration. Same was found by Sivagurunathan et al. (2015) where butyrate reduced concentration from 5.86 g/L to 4.67 g/L when pH shifted from 6.5 to 5.5. Consequently, biohydrogen production may be inhibitted due to substrate depletion or partial pressure of hydrogen exerted on cells by its the high accumulated volume.

FIGURE 18 – KINETICS OF SUBSTRATE CONSUMPTION AND PRODUCT FORMATION BY LPB AH8



SOURCE: The Author (2020)

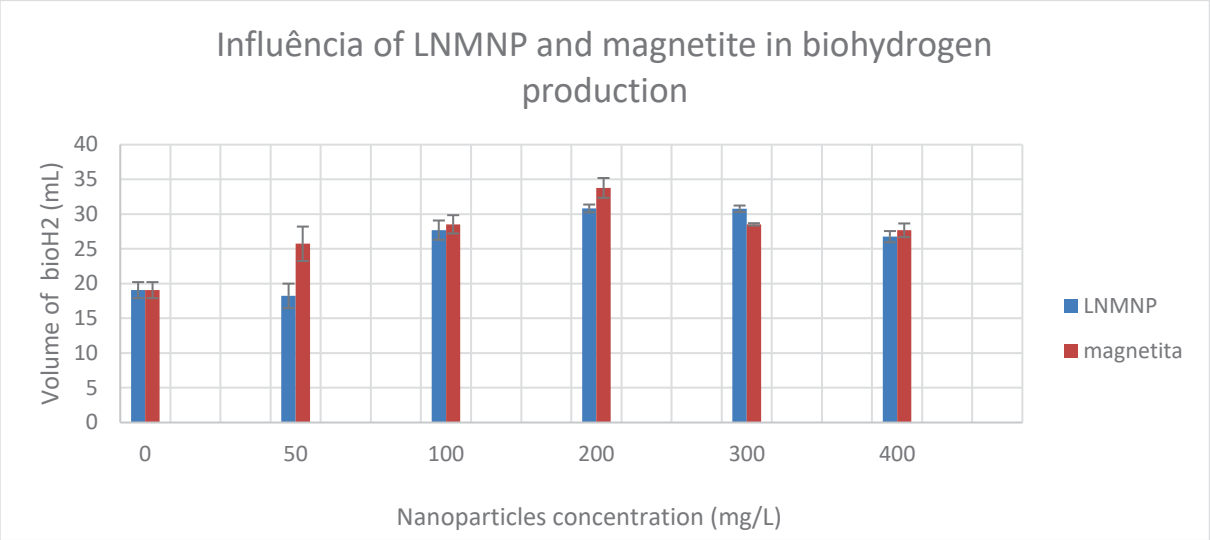
5.3.4 Evaluation of the presence of nanoparticles in media

It is noticeable in FIGURE 19 that there was a substantial increase in biohydrogen production after the addition of iron nanoparticles to the fermentation medium. There are several explanations reported in several studies indicating that iron binds to the active site of [Fe-Fe] hydrogenases accelerating the process of reducing hydrogen carriers to produce molecular hydrogen. Nanoparticles act comparatively to bridges formed between microorganisms, conducting electrons between acceptors and donors, increasing their metabolic flow (Mishra et al., 2018; Sakinah et al., 2017)

The metals released in the fermentation process by nanoparticles act in a way that the catalytic activity is increased in several studies because these metals act as enzymatic cofactors due to their very large surface area (Mishra et al., 2018; Taherdanak et al., 2015). On the other hand, Hsieh et al. (2016) analyzed the gene expression of *Clostridium pasterianum* and observed that addition of nanoparticles was not related to enzymatic activity improvement even though it was also found a stimulation and increase in biohydrogen production caused by the enhancement of electron transfer between hydrogenases and ferredoxyn. In addition to its catalytic activities, it is important to note that metals are needed in small quantities in dark

fermentation processes in order to stimulate bacterial growth and other processes related to anaerobic fermentation (Elbeshbishy et al., 2017; Han et al., 2011; Sakinah et al. 2017).

FIGURE 19 - COMPARISON OF BIOHYDROGEN PRODUCTION BY LPB AH8 WITH NANOPARTICLES AND THE CONTROL TEST



SOURCE: The author (2020)

Han et al. (2011) found in their studies that iron increased biohydrogen productivity of the utilized consortia. In their study with nickel and hematite nanoparticles, Gadhe et al. (2015) managed to increase the productivity of biohydrogen by 44% with 200 $mg.L^{-1}$ of hematite from residual distillation water with a pool of anaerobic reactor sludge. The nanoparticle concentration is equivalent to that found in this work for the LPB AH8 consortium, however the increase in productivity using this consortium was greater than that obtained by Gadhe, as can be seen in TABLE 7.

This increase may be probably attributed to iron's capability of inducing the expression of ferredoxin with positively direct action over hydrogenases (Chen et al. (2005); Elbeshbishy et al. (2017); Gadhe et al. (2015); Wu et al. (2017). Show et al. (2007) reported that nanoparticles formed "bridges" with effective extracellular electron transfer, resulting in conduiting and enhancing electrons transfer between a complex of cells. Wimonsong et al. (2014) also reported that Au catalysts form electron "sinks" at hydrogenases active sites, enhancing the electron affinity and promoting protons reduction to hydrogen. According to Lin et al. (2016) and Zhang&Shen (2007),

microorganisms internalize the nanoparticles promoting hydrogenases synthesis and more effective activation since iron not only enhances but activates ferredoxin oxidoreductase due to its high surface area and quantum size effects.

Enhancement of biohydrogen production obtained in this study is higher than most studies already done in the past. Magnetite (LMNP) nanoparticles made with lignin increased biohydrogen production in 91% with an accumulated volume of 33.8 mL of biohydrogen while the lignin non-magnetic nanoparticles (LNMNP) also increased biohydrogen in 74.3% with an accumulated volume of 30.8 mL when compared to the control test which had no adding of nanoparticles and accumulated a volume of biohydrogen of 19 mL. Mohanraj&Kodhaiyolii (2014) reported in their study that green synthesized iron nanoparticles were more effective in enhancing biohydrogen production than ferrous iron. Thus, it may be assumed that there was some interaction between nanoparticles and the anaerobic consortium.

TABLE 7 - COMPARATIVE ENHANCEMENT IN BIOHYDROGEN PRODUCTION APPLYING NANOPARTICLES

Microorganisms	**Nanoparticle**	**Carbon source**	**Enhancement due to nanoparticle**	**Author**
Clostridium butyricum	Hematite	Sucrose	32,64%	Han et al. (2011)
Anaerobic seed sludge	Hematite	Distillery wastewater	44%	Gadhe et al. (2015)
Clostridium butyricum	Iron oxide	Glucose	38%	Beckers et al. (2013)
Clostridium pasteurianum	Magnetite	PYG (*peptone, yeast, glucose)*	24,9%	Hsieh et al. (2016)
Consortium dominated by *Clostridium butyricum*	Silver	Glucose	62%	Zhao et al. (2013)
Anaerobic mixed Consortium	Iron oxide	Glucose	34,38%	Sakinah et al. (2017)
LPB AH8	LNMNP LMNP - Magnetite	Soft Drink wastewater & CSL	74.3% 91%	This study

SOURCE: The author (2020)

It is also found that at certain concentrations, iron can inhibit the production of biohydrogen as well as alter the metabolic pathways of microorganisms. (Elbeshbishy et al., 2017; Lin et al., 2016; Vaňáčová et al., 2001). Even though iron has an enzyme activator factor, it may affect mechanisms involved in the transportation of ions and nutrients, tending to accumulate these compounds inside or outside the cells as clusters, interfering or even destructing cell membrane by oxidative stress due to the high concentration of iron nanoparticles (Lin et al., 2016).

This is the reason why in all tests carried out with hematite and magnetite, the production of biohydrogen begins to decrease at higher iron concentrations. It can be seen clearly from FIGURE 19 that there is a slight decrease in productivity for the consortia. Several authors found that high concentration of nanoparticles have inhibitory effects on biohydrogen production. Hsieh et al. (2016) also had decreases in biohydrogen production with magnetic hematite and TiO_2 nanoparticles in high concentrations. Taherdanak et al. (2015) decreased the production of biohydrogen when the concentration of Fe^0 nanoparticles in his study was superior to 50 mg/L. Mohanraj et al. (2016) observed that copper nanoparticles addition above 5.0 mg/L caused a sharp decrease in biohydrogen production.

It was found no significant difference regarding VFA's production before and after the addition of the iron nanoparticles. Apparently, butyric acid and acetic are consumed in some stages of the dark fermentation when there is no addition of nanoparticles in media. It may be assumed that the addition of nanoparticles not only enhances biohydrogen production but also stimulates the degradation of VFA's produced during the process. As well as the control test, no ethanol was produced. Same was found by Taherdanak et al. (2015) in his tests with Fe^0 nanoparticles for biohydrogen production, presuming that ethanolic and consequently propionic acid producing bacteria would have been inhibited since biohydrogen production was enhanced nevertheless.

Biohydrogen yields were given in terms of mLH_2 / $gCOD_{removed}$. The analysis showed that the addition of 200 mg / L of green nanoparticles improved the yields on 2.8 and 2.3 fold for magnetite and LNMNP, respectively. Values obtained in the control resulted in approximately 180.7 mLH_2 / $gCOD_{removed}$ while magnetite and LNMNP improved yields to approximately 506 mLH_2 / $gCOD_{removed}$ and 410 mLH_2 / $gCOD_{removed}$, respectively.

Mishra et al. (2018) also enhanced the yield of biohydrogen production in his study using palm oil mill effluent (POME) in 1.51 and 1.67 fold using nickel and cobalt nanoparticles, respectively. Gadhe et al. (2015a) attributed the enhancement of 62% in biohydrogen yields from distillery wastewater to the enhancement on ferredoxin oxidoreductase and hydrogenase activity due to the combination of Fe_2O_3 and NiO nanoparticles. Gadhe et al. (2015b) also attributed to the same reasons the 27% enhancement on biohydrogen yield from dairy wastewater with the coaddition of hematite and nickel nanoparticles.

5.4 CONCLUSIONS

The consortium used achieved high yields with and without the increment of green nanoparticles. The increase in biohydrogen production due to the addition of nanoparticles was expected since iron is a directly bonded to hydrogenases activity. High concentrations of nanoparticles inhibited microbial activity. It may be assumed that the increase in production happened due to hydrogenases improvement. Once the influence of nanoparticles was not kinetically measured and just comparative, a deeper study should be employed to further determine if these nanoparticles decrease the lag phase. A gene expression of hydrogenase may also be conducted to prove the interaction between biohydrogen and green nanoparticles.

Both soft drink's wastewater and corn steep liquor provided enough nutrients to carry a successful biohydrogen dark fermentation and the removal of organic load from these residues. Even though these are low-cost residues that may not only decrease waste disposal in the environment but also facilitate biohydrogen production economically in the future, it is important to highlight that its by-products will require physical treatment since, especially the effluents and separation of carbonic gas from the final hydrogen produced. Corn steep liquor is a promising carbon and nitrogen source that must be studied due to high yields achieved that may be attributed to it.

REFERENCES

AADIL, K. R.; BARAPATRE, A.; MEENA, A. S.; JHA, H. Hydrogen peroxide sensing and cytotoxicity activity of Acacia lignin stabilized silver nanoparticles. **International Journal of Biological Macromolecules**, v. 82, p. 39–47, 2016. Elsevier B.V.

ABBASI KAJANI, A.; BORDBAR, A. Biogenic magnetite nanoparticles: A potent and environmentally benign agent for efficient removal of azo dyes and phenolic contaminants from water. **Journal of Hazardous Materials**, v. 366, p. 268–274, 2019. Elsevier B.V.

ABDEL-FATAH, M. A.; SHERIF, H. O.; HAWASH, S. I. Design parameters for waste effluent treatment unit from beverages production. **Ain Shams Engineering Journal**, v. 8, n. 3, p. 305–310, 2017. Ain Shams University. Disponível em: <http://dx.doi.org/10.1016/j.asej.2016.04.008>. .

ABUBACKAR, H. N.; KESKIN, T.; YAZGIN, O.; et al. Biohydrogen production from autoclaved fruit and vegetable wastes by dry fermentation under thermophilic condition. **International Journal of Hydrogen Energy**, v. 44, n. 34, p. 18776–18784, 2019. Elsevier Ltd.

ALRIOLS, M. G.; GARCÍA, A.; LABIDI, J. Combined organosolv and ultrafiltration lignocellulosic biorefinery process. **Chemical Engineering Journal**, v. 157, p. 113–120, 2010.

ALVARADO-CUEVAS, Z.; ACEVEDO, L. G. O.; SALAS, J. T. O.; LEÓN-RODRÍGUEZ, A. DE. Nitrogen sources impact hydrogen production by Escherichia coli using cheese whey as substrate. **New Biotechnology**, v. 00, n. 00, p. 2–7, 2013.

ARANTES, M. K.; SEQUINEL, R.; ALVES, H. J.; et al. Improvement of biohydrogen production from brewery wastewater: Evaluation of inocula, support and reactor. **International Journal of Hydrogen Energy**, v. 45, n. 8, p. 5216–5226, 2020.

BALCH, W. E.; FOX, G. E.; MAGRUM, L. J.; WOESE, C. R.; WOLFE, R. S. Methanogens : Reevaluation of a Unique Biological Group. **Microbiological Reviews**, v. 43, n. 2, p. 260–296, 1979.

BAO, M. D.; SU, H. J.; TAN, T. W. Dark fermentative bio-hydrogen production : Effects of substrate pre-treatment and addition of metal ions or L -cysteine. **Fuel**, v. 112, p. 38–44, 2013.

BARKER, H. A. Amino Acid Degradation by Anaerobic Bacteria. **Annual Review of Biochemistry**, v. 50, n. 1, p. 23–40, 1981.

BECKERS, L.; HILIGSMANN, S.; LAMBERT, S. D.; HEINRICHS, B.; THONART, P. Improving effect of metal and oxide nanoparticles encapsulated in porous silica on fermentative biohydrogen production by Clostridium butyricum. **Bioresource Technology**, v. 133, p. 109–117, 2013. Elsevier Ltd.

BISHNOI, S.; KUMAR, A.; SELVARAJ, R. Facile synthesis of magnetic iron oxide nanoparticles using inedible Cynometra ramiflora fruit extract waste and their photocatalytic degradation of methylene blue dye. **Materials Research Bulletin**, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.materresbull.2017.08.040>. .

BITTENCOURT, E.; LARROCHE, C.; CRISTINE, A.; et al. Bioresource Technology Economic process to produce biohydrogen and volatile fatty acids by a mixed culture using vinasse from sugarcane ethanol industry as nutrient source. **BIORESOURCE TECHNOLOGY**, v. 159, p. 380–386, 2014. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2014.02.042>. .

CAI, G.; JIN, B.; SAINT, C.; MONIS, P. Metabolic flux analysis of hydrogen production network by Clostridium butyricum W5 : Effect of pH and glucose concentrations. **International Journal of Hydrogen Energy**, v. 35, n. 13, p. 6681–6690, 2010. Elsevier Ltd. Disponível em:

<http://dx.doi.org/10.1016/j.ijhydene.2010.04.097>. .
CARRILLO, I.; MENDONÇA, R. T.; AGO, M.; ROJAS, O. J. Comparative study of cellulosic components isolated from different Eucalyptus species. **Cellulose**, v. 25, n. 2, p. 1011–1029, 2018.
CARVALHO, F.; PRAZERES, A. R.; RIVAS, J. Science of the Total Environment Cheese whey wastewater : Characterization and treatment. **Science of the Total Environment, The**, v. 445–446, p. 385–396, 2013. Elsevier B.V. Disponível em: <http://dx.doi.org/10.1016/j.scitotenv.2012.12.038>. .
CAVALLI-SFORZA, L. L.; EDWARDS, A. W. Phylogenetic analysis. Models and estimation procedures. **American Journal of Human Genetics**, v. 19, n. 3, p. 233–257, 1967.
CHEN, W.; TSENG, Z.; LEE, K.; CHANG, J. Fermentative hydrogen production with Clostridium butyricum CGS5 isolated from anaerobic sewage sludge. , v. 30, p. 1063–1070, 2005.
CHU, C.; TUNG, L.; LIN, C. Effect of substrate concentration and pH on biohydrogen production kinetics from food industry wastewater by mixed culture. **International Journal of Hydrogen Energy**, v. 38, n. 35, p. 15849–15855, 2013. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2013.07.088>. .
CISNEROS DE LA CUEVA, S.; ALVAREZ GUZMÁN, C. L.; BALDERAS HERNÁNDEZ, V. E.; DE LEÓN RODRÍGUEZ, A. Optimization of biohydrogen production by the novel psychrophilic strain N92 collected from the Antarctica. **International Journal of Hydrogen Energy**, v. 43, n. 30, p. 13798–13809, 2018.
CONSTANT, S.; BASSET, C.; DUMAS, C.; et al. Reactive organosolv lignin extraction from wheat straw: Influence of Lewis acid catalysts on structural and chemical properties of lignins. **Industrial Crops and Products**, v. 65, p. 180–189, 2015.
D'ESTE, M.; ALVARADO-MORALES, M.; ANGELIDAKI, I. Amino acids production focusing on fermentation technologies – A review. **Biotechnology Advances**, v. 36, n. 1, p. 14–25, 2018. Elsevier. Disponível em: <http://dx.doi.org/10.1016/j.biotechadv.2017.09.001>. .
DÇBOWSKI, M.; KORZENIEWSKA, E.; FILIPKOWSKA, Z.; ZIELINSKI, M.; KWIATKOWSKI, R. Possibility of hydrogen production during cheese whey fermentation process by different strains of psychrophilic bacteria. **internationa l Journal of Hydrogen Energy**, v. 9, p. 1972–1978, 2014.
DURRE, P. **Handbook of Clostridia**. 2005.
ELBESHBISHY, E.; DHAR, B. R.; NAKHLA, G.; LEE, H. A critical review on inhibition of dark biohydrogen fermentation. **Renewable and Sustainable Energy Reviews**, v. 79, n. October 2015, p. 656–668, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.rser.2017.05.075>. .
ENGLIMAN, N. S.; ABDUL, P. M.; WU, S. Y.; JAHIM, J. M. Influence of iron (II) oxide nanoparticle on biohydrogen production in thermophilic mixed fermentation. **International Journal of Hydrogen Energy**, v. 42, n. 45, p. 27482–27493, 2017. Elsevier Ltd.
ESAKKIMUTHU, T.; SIVAKUMAR, D.; AKILA, S. Application of nanoparticles in wastewater treatment TREATMENT. , , n. May, 2017.
ESCALANTE, H.; CASTRO, L.; AMAYA, M. P.; JAIMES, L. Anaerobic digestion of cheese whey : Energetic and nutritional potential for the dairy sector in developing countries. **Waste Management**, v. 71, p. 711–718, 2018. Disponível em: <https://doi.org/10.1016/j.wasman.2017.09.026>. .
ETCHEBEHERE, C.; CARRER, B.; RU, P.; et al. ScienceDirect Role of homo-and

heterofermentative lactic acid bacteria on hydrogen-producing reactors operated with cheese whey wastewater. , v. 0, 2015.
FARIZOGLU, B.; KESKINLER, B.; YILDIZ, E.; NUHOGLU, A. Cheese whey treatment performance of an aerobic jet loop membrane bioreactor. **Process Biochemistry**, v. 39, p. 2283–2291, 2004.
FAZLZADEH, M.; RAHMANI, K.; ZAREI, A.; et al. A novel green synthesis of zero valent iron nanoparticles (NZVI) using three plant extracts and their efficient application for removal of Cr(VI) from aqueous solutions. **Advanced Powder Technology**, v. 28, n. 1, p. 122–130, 2017. The Society of Powder Technology Japan.
FERCHICHI, M.; CRABBE, E.; GIL, G. H.; HINTZ, W.; ALMADIDY, A. Influence of initial pH on hydrogen production from cheese whey. **Journal of Biotechnology**, v. 120, n. 4, p. 402–409, 2005.
FLAUZINO NETO, W. P.; SILVÉRIO, H. A.; DANTAS, N. O.; PASQUINI, D. Extraction and characterization of cellulose nanocrystals from agro-industrial residue - Soy hulls. **Industrial Crops and Products**, v. 42, n. 1, p. 480–488, 2013. Elsevier B.V.
GADHE, A.; SONAWANE, S. S.; VARMA, M. N. ScienceDirect Influence of nickel and hematite nanoparticle powder on the production of biohydrogen from complex distillery wastewater in batch fermentation. **International Journal of Hydrogen Energy**, v. 40, n. 34, p. 10734–10743, 2015a. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2015.05.198>. .
GADHE, A.; SONAWANE, S. S.; VARMA, M. N. ScienceDirect Enhancement effect of hematite and nickel nanoparticles on biohydrogen production from dairy wastewater. **International Journal of Hydrogen Energy**, v. 40, n. 13, p. 4502–4511, 2015b. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2015.02.046>. .
GHIMIRE, A.; FRUNZO, L.; PONTONI, L.; et al. Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. **Journal of Environmental Management**, v. 152, p. 43–48, 2015. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.jenvman.2014.12.049>. .
GIOANNIS, G. DE; FRIARGIU, M.; MASSI, E.; MUNTONI, A.; POLETTINI, A. ScienceDirect Biohydrogen production from dark fermentation of cheese whey : Influence of pH. **International Journal of Hydrogen Energy**, v. 39, n. 36, p. 20930–20941, 2014. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2014.10.046>. .
HALL, W. L.; MILLWARD, D. J.; LONG, S. J.; MORGAN, L. M. Casein and whey exert different effects on plasma amino acid profiles , gastrointestinal hormone secretion and appetite. **British Journal of Nutrition**, v. 89, p. 239–248, 2003.
HALLENBECK, P. C.; ABO-HASHESH, M.; GHOSH, D. Bioresource Technology Strategies for improving biological hydrogen production. **Bioresource Technology**, v. 110, p. 1–9, 2012. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2012.01.103>. .
HAMILTON, C.; CALUSINSKA, M.; BAPTISTE, S.; et al. ScienceDirect Effect of the nitrogen source on the hydrogen production metabolism and hydrogenases of Clostridium butyricum CWBI1009. **International Journal of Hydrogen Energy**, v. 43, n. 11, p. 5451–5462, 2017. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.ijhydene.2017.12.162>. .
HAMILTON, C.; CALUSINSKA, M.; BAPTISTE, S.; et al. Effect of the nitrogen source on the hydrogen production metabolism and hydrogenases of Clostridium butyricum

CWBI1009. **International Journal of Hydrogen Energy**, v. 43, n. 11, p. 5451–5462, 2018. Elsevier Ltd.
HAN, H.; CUI, M.; WEI, L.; YANG, H.; SHEN, J. Bioresource Technology Enhancement effect of hematite nanoparticles on fermentative hydrogen production. **Bioresource Technology**, v. 102, n. 17, p. 7903–7909, 2011. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2011.05.089>. .
HIDALGO, D.; MARTÍN-MARROQUÍN, J. M. **ADDING SUSTAINABILITY TO**. Elsevier Inc., 2019.
HITIT, Z. Y.; LAZARO, C. Z.; HALLENBECK, P. C. Hydrogen production by co-cultures of Clostridium butyricum and Rhodospeudomonas palustris: Optimization of yield using response surface methodology. **International Journal of Hydrogen Energy**, v. 42, n. 10, p. 6578–6589, 2017. Elsevier Ltd.
HOFER, A. A.; HAUER, S.; KROLL, P.; HERWIG, C. Graphical abstract. **Process Biochemistry**, v. 70, p. 20–28, 2018. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.procbio.2018.04.008>. .
HOFER, A.; HAUER, S.; KROLL, P.; FRICKE, J.; HERWIG, C. In-depth characterization of the raw material corn steep liquor and its bioavailability in bioprocesses of Penicillium chrysogenum. **Process Biochemistry**, p. 1–33, 2018. Elsevier Ltd.
HOLLADAY, J. D.; HU, J.; KING, D. L.; WANG, Y. An overview of hydrogen production technologies. **Catalysis Today**, jan. 2009. Elsevier.
HSIEH, P.; LAI, Y.; CHEN, K.; HUNG, C. ScienceDirect Explore the possible effect of TiO 2 and magnetic hematite nanoparticle addition on biohydrogen production by Clostridium pasteurianum based on gene expression measurements. **International Journal of Hydrogen Energy**, v. 41, n. 46, p. 21685–21691, 2016. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2016.06.197>. .
HUNG, C.; CHANG, YI-TANG; CHANG, YU-JIE. Bioresource Technology Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems – A review. **Bioresource Technology**, v. 102, n. 18, p. 8437–8444, 2011. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2011.02.084>. .
HYE, J.; LEE, D. S.; PARK, D.; CHOE, W.; PARK, J. M. Optimization of key process variables for enhanced hydrogen production by Enterobacter aerogenes using statistical methods. **Bioresource Technology**, v. 99, p. 2061–2066, 2008.
ISLA, M. A.; COMELLI, R. N.; SELUY, L. G. Biore source Tec hnology Wastewater from the soft drinks industry as a source for bioethanol production. **Bioresource Technology**, v. 136, p. 140–147, 2013. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2013.02.089>. .
ISROI; ISHOLA, M.; MILLATI, R.; et al. Structural Changes of Oil Palm Empty Fruit Bunch (OPEFB) after Fungal and Phosphoric Acid Pretreatment. **Molecules**, v. 17, n. 12, p. 14995–15012, 2012.
J, F. Confidence Limits on Phylogenies : An Approach Using the Bootstrap Author (s): Joseph Felsenstein Stable URL : http://www.jstor.org/stable/2408678 . **Evolution**, v. 39, n. 4, p. 783–791, 1985.
KAMALASKAR, L.; KAPSE, N.; PORE, S.; DHAKEPHALKAR, ANITA P.; et al. Genome sequence and gene expression studies reveal novel hydrogenases mediated hydrogen production by Clostridium biohydrogenum sp. nov., MCM B-509T. **International Journal of Hydrogen Energy**, v. 41, n. 28, p. 11990–11999, 2016. Elsevier Ltd.
KAMALASKAR, L.; KAPSE, N.; PORE, S.; DHAKEPHALKAR, ANITA P; et al.

ScienceDirect Genome sequence and gene expression studies reveal novel hydrogenases mediated hydrogen production by Clostridium biohydrogenum sp . nov .,. **International Journal of Hydrogen Energy**, p. 1–10, 2016. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2016.05.101>. .
KANCHANASUTA, S.; PROMMEENATE, P. ScienceDirect Stability of Clostridium butyricum in biohydrogen production from non-sterile food waste. **International Journal of Hydrogen Energy**, p. 1–12, 2016. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2016.09.111>. .
KAPDAN, I. K.; KARGI, F. Bio-hydrogen production from waste materials. **Enzyme and Microbial Technology**, v. 38, p. 569–582, 2006.
KARPAGAVINAYAGAM, P.; VEDHI, C. Green synthesis of iron oxide nanoparticles using Avicennia marina flower extract. **Vacuum**, v. 160, p. 286–292, 2019. Elsevier Ltd.
KIM, S. B.; LEE, S. J.; LEE, J. H.; et al. Pretreatment of rice straw with combined process using dilute sulfuric acid and aqueous ammonia. **Biotechnology for Biofuels**, v. 6, n. 1, p. 1–11, 2013.
KIM, S.; ROLLY, R.; SEOK, J.; KIM, S. Optimization of dilute acid and enzymatic hydrolysis for dark fermentative hydrogen production from the empty fruit bunch of oil palm ScienceDirect Optimization of dilute acid and enzymatic hydrolysis for dark fermentative hydrogen production from the emp. **International Journal of Hydrogen Energy**, , n. September, 2018. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.ijhydene.2018.08.022>. .
KLUGE, A. G.; FARRIS, J. S. Society of Systematic Biologists Quantitative Phyletics and the Evolution of Anurans QUANTITATIVE PHYLETICS AND THE EVOLUTION OF ANURANS. **Society**, v. 18, n. 1, p. 1–32, 2011.
KOPSAHELIS, K. P. S. A.; KORNAROS, C. Z. M. Effect of pH on Continuous Biohydrogen Production from End- of-Life Dairy Products (EoL-DPs) via Dark Fermentation. **Waste and Biomass Valorization**, v. 7, n. 4, p. 753–764, 2016. Springer Netherlands.
KREGIEL, D. Health safety of soft drinks: Contents, containers, and microorganisms. **BioMed Research International**, v. 2015, 2015.
KRISHNAN, S.; SINGH, L.; SAKINAH, M.; et al. Process enhancement of hydrogen and methane production from palm oil mill effluent using two-stage thermophilic and mesophilic fermentation. **International Journal of Hydrogen Energy**, v. 41, n. 30, p. 12888–12898, 2016. Elsevier Ltd.
KUMAR, G.; BAKONYI, P.; SIVAGURUNATHAN, P.; et al. Enhanced biohydrogen production from beverage industrial wastewater using external nitrogen sources and bioaugmentation with facultative anaerobic strains. **Journal of Bioscience and Bioengineering**, v. 120, n. 2, p. 155–160, 2015.
KUMAR, S.; STECHER, G.; LI, M.; KNYAZ, C.; TAMURA, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. **Molecular Biology and Evolution**, v. 35, n. 6, p. 1547–1549, 2018.
KUSMARDINI, D.; PRASETYO, J.; HUDIYONO, S. The Effectiveness of Bio-Delignification Empty Fruit Bunch of Palm Oil by Aspergillus fumigatus : Targeting for Bio-Hydrogen Production. , v. 12, n. 3, p. 411–427, 2016.
LAY, C. H.; VO, T. P.; LIN, P. Y.; et al. Anaerobic hydrogen and methane production from low-strength beverage wastewater. **International Journal of Hydrogen Energy**, v. 44, n. 28, p. 14351–14361, 2019. Elsevier Ltd.
LEE, D.; SHOW, K.; SU, A. Bioresource Technology Dark fermentation on biohydrogen production : Pure culture. , v. 102, p. 8393–8402, 2011.

LEO, A. DE; DAVILA-VAZQUEZ, G.; ALATRISTE-MONDRAGO, F. Fermentative hydrogen production in batch experiments using lactose , cheese whey and glucose : Influence of initial substrate concentration and pH. , v. 33, p. 4989–4997, 2008.
LI, W.; CHENG, C.; CAO, G.; REN, N. Enhanced biohydrogen production from sugarcane molasses by adding Ginkgo biloba leaves. **Bioresource Technology**, v. 298, n. 73, p. 122523, 2020. Elsevier.
LI, X.; XU, W.; YANG, J.; et al. SC. **Animal Nutrition Journal**, 2016. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.aninu.2016.09.003>. .
LIN, P.; WHANG, L.; WU, Y.; REN, W.; HSIAO, C. Biological hydrogen production of the genus Clostridium : Metabolic study and mathematical model simulation. , v. 32, p. 1728–1735, 2007.
LIN, P. Y.; WHANG, L. M.; WU, Y. R.; et al. Biological hydrogen production of the genus Clostridium: Metabolic study and mathematical model simulation. **International Journal of Hydrogen Energy**, v. 32, n. 12, p. 1728–1735, 2007.
LIN, R.; CHENG, J.; DING, L.; et al. Bioresource Technology Enhanced dark hydrogen fermentation by addition of ferric oxide nanoparticles using Enterobacter aerogenes. **BIORESOURCE TECHNOLOGY**, v. 207, p. 213–219, 2016. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2016.02.009>. .
LIU, B.; JIN, Y.; WANG, Z.; XING, D.; MA, C. ScienceDirect Enhanced photo-fermentative hydrogen production of Rhodopseudomonas sp . nov . strain A7 by the addition of TiO 2 , ZnO and SiC nanoparticles. **International Journal of Hydrogen Energy**, v. 42, n. 29, p. 18279–18287, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2017.04.147>. .
LIU, C.; ZHENG, J.; WU, S.; CHU, C. ScienceDirect Fermentative hydrogen production potential from washing wastewater of beverage production process. **International Journal of Hydrogen Energy**, v. 41, n. 7, p. 4466–4473, 2015a. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2015.08.079>. .
LIU, C.; ZHENG, J.; WU, S.; CHU, C. ScienceDirect Fermentative hydrogen production potential from washing wastewater of beverage production process. **International Journal of Hydrogen Energy**, p. 1–8, 2015b. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2015.08.079>. .
LIU, G.; SHEN, J. Effects of Culture and Medium Conditions on Hydrogen Production from Starch Using Anaerobic Bacteria. **Journal of Bioscience and Bioengineering**, v. 98, n. 4, p. 251–256, 2004.
LIU, X.; WANG, X.; XU, J.; et al. Citric acid production by Yarrowia lipolytica SWJ-1b using corn steep liquor as a source of organic nitrogen and vitamins. **Industrial Crops & Products**, v. 78, p. 154–160, 2015. Elsevier B.V. Disponível em: <http://dx.doi.org/10.1016/j.indcrop.2015.10.029>. .
LOH, S. K. The potential of the Malaysian oil palm biomass as a renewable energy source. **Energy Conversion and Management**, v. 141, p. 285–298, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.enconman.2016.08.081>. .
LU, Z.; LI, J.; ZHU, A.; et al. Catalytic hydrolysis of ammonia borane via magnetically recyclable copper iron nanoparticles for chemical hydrogen storage. **International Journal of Hydrogen Energy**, v. 38, n. 13, p. 5330–5337, 2013. Elsevier Ltd.
MADDIPATI, P.; ATIYEH, H. K.; BELLMER, D. D.; HUHNKE, R. L. Bioresource Technology Ethanol production from syngas by Clostridium strain P11 using corn steep liquor as a nutrient replacement to yeast extract. **Bioresource Technology**, v. 102, n. 11, p. 6494–6501, 2011. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2011.03.047>. .
MAHATO, R. K.; KUMAR, D.; RAJAGOPALAN, G. Biohydrogen production from fruit

waste by Clostridium strain BOH3. **Renewable Energy**, v. 153, p. 1368–1377, 2020. Elsevier Ltd.
MALIK, S. N.; PUGALENTHI, V.; VAIDYA, A. N.; GHOSH, P. C. Kinetics of nano-catalysed dark fermentative hydrogen production from distillery wastewater. **Energy Procedia**, v. 54, p. 417–430, 2014. Elsevier B.V. Disponível em: <http://dx.doi.org/10.1016/j.egypro.2014.07.284>. .
MARBÁN, G.; VALDÉS-SOLÍS, T. Towards the hydrogen economy? **International Journal of Hydrogen Energy**, v. 32, n. 12, p. 1625–1637, 2007. Elsevier Ltd.
MARI, A. G.; ANDREANI, C. L.; TONELLO, T. U.; et al. Biohydrogen and biomethane production from cassava wastewater in a two-stage anaerobic sequencing batch biofilm reactor. **International Journal of Hydrogen Energy**, v. 45, n. 8, p. 5165–5174, 2020.
MARTINEZ-BURGOS, W. J.; SYDNEY, E. B.; BRAR, S. K.; et al. The effect of hydrolysis and sterilization in biohydrogen production from cassava processing wastewater medium using anaerobic bacterial consortia. **International Journal of Hydrogen Energy**, v. 44, n. 47, p. 25551–25564, 2019.
MARTINEZ-BURGOS, W. J.; SYDNEY, E. B.; DE PAULA, D. R.; et al. Biohydrogen production in cassava processing wastewater using microbial consortia: Process optimization and kinetic analysis of the microbial community. **Bioresource Technology**, v. 309, p. 123331, 2020. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.biortech.2020.123331>. .
MARTINEZ-BURGOS, W.; SYDNEY, E.; BRAR, S.; et al. The effect of hydrolysis and sterilization in biohydrogen production from cassava processing wastewater medium using anaerobic bacterial consortia. **International jorurnal of hydrogen energy**, v. 4, n. 25551–25564, 2019.
MISHRA, P.; THAKUR, S.; MAHAPATRA, D. M.; et al. Impacts of nano-metal oxides on hydrogen production in anaerobic digestion of palm oil mill effluent – A novel approach ScienceDirect Impacts of nano-metal oxides on hydrogen production in anaerobic digestion of palm oil mill effluent e A novel approach. **International Journal of Hydrogen Energy**, , n. January, 2018. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.ijhydene.2017.12.108>. .
MOHANRAJ, S.; ANBALAGAN, K.; RAJAGURU, P. ScienceDirect Effects of phytogenic copper nanoparticles on fermentative hydrogen production by Enterobacter cloacae and Clostridium acetobutylicum. **International Journal of Hydrogen Energy**, v. 41, n. 25, p. 10639–10645, 2016. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2016.04.197>. .
MOHANRAJ, S.; KODHAIYOLII, S. ScienceDirect Phytosynthesized iron oxide nanoparticles and ferrous iron on fermentative hydrogen production using Enterobacter cloacae : Evaluation and comparison of the effects. **International Journal of Hydrogen Energy**, v. 39, n. 23, p. 11920–11929, 2014. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2014.06.027>. .
MORENO, R.; FIERRO, J.; FERNÁNDEZ, C.; et al. Biohydrogen production from lactose : influence of substrate and nitrogen concentration. , v. 3330, 2015. Disponível em: <https://doi.org/10.1080/09593330.2015.1032365>. .
NASR, M.; TAWFIK, A.; OOKAWARA, S.; et al. Journal of Industrial and Engineering Chemistry Continuous biohydrogen production from starch wastewater via sequential dark-photo fermentation with emphasize on maghemite nanoparticles. **Journal of Industrial and Engineering Chemistry**, v. 21, p. 500–506, 2015. The Korean Society of Industrial and Engineering Chemistry. Disponível em: <http://dx.doi.org/10.1016/j.jiec.2014.03.011>. .

NATH, K.; DAS, D. Improvement of fermentative hydrogen production: Various approaches. **Applied Microbiology and Biotechnology**, out. 2004.
OGUERI, N.; VINCENT, I. I. Prevalence and risk of heterotrophic microorganisms in a carbonated soft drink factory. **African Journal of Microbiology Research**, v. 2015, n. 6, p. 245–253, 2017.
PAN, C.; FAN, Y.; ZHAO, P.; HOU, H. Fermentative hydrogen production by the newly isolated Clostridium beijerinckii Fanp3. **internationa l jo urnal of hydrogen energy**, v. 33, p. 5383–5391, 2008a.
PAN, C.; FAN, Y.; ZHAO, P.; HOU, H. Fermentative hydrogen production by the newly isolated Clostridium beijerinckii Fanp3. **International Journal of Hydrogen Energy**, v. 33, n. 20, p. 5383–5391, 2008b. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2008.05.037>. .
PANDEY, ANJANA; GUPTA, K.; PANDEY, ASHUTOSH. ScienceDirect Effect of nanosized TiO 2 on photofermentation by Rhodobacter sphaeroides NMBL-02. **Biomass and Bioenergy**, v. 72, p. 273–279, 2014. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biombioe.2014.10.021>. .
PATTRA, S.; SANGYOKA, S.; BOONMEE, M.; REUNGSANG, A. Bio-hydrogen production from the fermentation of sugarcane bagasse hydrolysate by Clostridium butyricum. **International Journal of Hydrogen Energy**, v. 33, p. 5256–5265, 2008.
PEIXOTO, G.; SAAVEDRA, N. K.; VARESCHE, M. B. A.; ZAIAT, M. Hydrogen production from soft-drink wastewater in an upflow anaerobic packed-bed reactor. **International Journal of Hydrogen Energy**, v. 36, n. 15, p. 8953–8966, 2011. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2011.05.014>. .
PRABAKAR, D.; MANIMUDI, V. T.; SUVETHA K, S.; et al. Advanced biohydrogen production using pretreated industrial waste: Outlook and prospects. **Renewable and Sustainable Energy Reviews**, v. 96, n. August, p. 306–324, 2018. Elsevier Ltd.
SAADY, N. M. C. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation : Unresolved challenge. **International jorurnal of hydrogen energy**, v. 8, 2013.
SAID, S.; IMAI, T.; SALAH, M.; et al. ScienceDirect Identification of factors that accelerate hydrogen production by Clostridium butyricum RAK25832 using casamino acids as a nitrogen source. **International Journal of Hydrogen Energy**, p. 1–14, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2017.08.171>. .
SAKINAH, N.; MOHAMED, P.; WU, S. ScienceDirect Influence of iron (II) oxide nanoparticle on biohydrogen production in thermophilic mixed fermentation. **International Journal of Hydrogen Energy**, v. 42, n. 45, p. 27482–27493, 2017. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.ijhydene.2017.05.224>. .
SEPPA, J. J. Fermentative hydrogen production by Clostridium butyricum and Escherichia coli in pure and cocultures. , v. 6, p. 3–10, 2011.
SHAH, A. T.; FAVARO, L.; ALIBARDI, L.; et al. Bacillus sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste. **Applied Energy**, v. 176, p. 116–124, 2016. Elsevier Ltd.
SHARMA, P.; MELKANIA, U. Enhancement effect of amino acids on hydrogen production from organic fraction of municipal solid waste using co-culture of Escherichia coli and Enterobacter aerogenes. **Energy conversion and Management**, v. 163, n. February, p. 260–267, 2018. Elsevier.
SHOW, K. Y.; ZHANG, Z. P.; TAY, J. H.; et al. Production of hydrogen in a granular sludge-based anaerobic continuous stirred tank reactor. **International Journal of Hydrogen Energy**, v. 32, n. 18, p. 4744–4753, 2007.

SIKORA, A.; BLASZCZYK, M.; JURKOWSKI, M.; ZIELENKIEWICZ, U. Lactic acid bacteria in hydorgen-producing Consortia: On pupose or by coincidencia? **Lactic Acid Bacteria – R & D for Food, Health and Livestock Purposes been**. p.487–513, 2013.

SINGH, K. K.; SENAPATI, K. K.; SARMA, K. C. Journal of Environmental Chemical Engineering Synthesis of superparamagnetic Fe 3 O 4 nanoparticles coated with green tea polyphenols and their use for removal of dye pollutant from aqueous solution. **Journal of Environmental Chemical Engineering**, v. 5, n. 3, p. 2214–2221, 2017. Elsevier. Disponível em: <http://dx.doi.org/10.1016/j.jece.2017.04.022>. .

SINGH, S. K.; DHEPE, P. L. Isolation of lignin by organosolv process from different varieties of rice husk: Understanding their physical and chemical properties. **Bioresource Technology**, v. 221, p. 310–317, 2016. Elsevier Ltd.

SIVAGURUNATHAN, P.; LIN, C. Y. Biohydrogen Production From Beverage Wastewater Using Selectively Enriched Mixed Culture. **Waste and Biomass Valorization**, v. 0, n. 0, p. 0, 2019. Springer Netherlands. Disponível em: <http://dx.doi.org/10.1007/s12649-019-00606-z>. .

SIVAGURUNATHAN, P.; LIN, C. Y. Biohydrogen Production From Beverage Wastewater Using Selectively Enriched Mixed Culture. **Waste and Biomass Valorization**, v. 11, n. 3, p. 1049–1058, 2020. Springer Netherlands.

SIVAGURUNATHAN, P.; SEN, B.; LIN, C. High-rate fermentative hydrogen production from beverage wastewater. **Applied Energy**, v. 147, p. 1–9, 2015. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.apenergy.2015.01.136>. .

SKONIECZNY, M. T.; YARGEAU, V. Biohydrogen production from wastewater by Clostridium beijerinckii : Effect of pH and substrate concentration. **International Journal of Hydrogen Energy**, v. 34, p. 3288–3294, 2009.

SREETHAWONG, T.; CHATSIRIWATANA, S. Hydrogen production from cassava wastewater using an anaerobic sequencing batch reactor : Effects of operational parameters , COD : N ratio , and organic acid composition. **International Journal of Hydrogen Energy**, v. 35, n. 9, p. 4092–4102, 2010. Elsevier Ltd.

SYDNEY, E. B.; DUARTE, E. R.; MARTINEZ-BURGOS, W. J.; et al. Development of short chain fatty acid-based artificial neuron network tools applied to biohydrogen production. **International Jorurnal of Hydrogen Energy**, v. 5, p. 5175–5181, 2020.

SYDNEY, E. B.; NOVAK, A. C.; ROSA, D.; et al. Screening and bioprospecting of anaerobic consortia for biohydrogen and volatile fatty acid production in a vinasse based medium through dark fermentation. **Process Biochemistry**, v. 67, n. November 2017, p. 1–7, 2018. Elsevier. Disponível em: <http://dx.doi.org/10.1016/j.procbio.2018.01.012>. .

TAHERDANAK, M.; ZILOUEI, H.; KARIMI, K. ScienceDirect Investigating the effects of iron and nickel nanoparticles on dark hydrogen fermentation from starch using central composite design. **International Journal of Hydrogen Energy**, v. 40, n. 38, p. 12956–12963, 2015a. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2015.08.004>. .

TAHERDANAK, M.; ZILOUEI, H.; KARIMI, K. ScienceDirect The effects of Fe 0 and Ni 0 nanoparticles versus Fe 2 þ and Ni 2 þ ions on dark hydrogen fermentation. **International Journal of Hydrogen Energy**, v. 41, n. 1, p. 167–173, 2015b. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2015.11.110>. .

THANWISED, P.; WIROJANAGUD, W.; REUNGSANG, A. Effect of hydraulic retention time on hydrogen production and chemical oxygen demand removal from tapioca wastewater using anaerobic mixed cultures in anaerobic baffled reactor (

ABR). **International Journal of Hydrogen Energy**, v. 37, n. 20, p. 15503–15510, 2012. Elsevier Ltd.
TOLEDANO, A.; GARCÍA, A.; MONDRAGON, I.; LABIDI, J. Lignin separation and fractionation by ultrafiltration. **Separation and Purification Technology**, v. 71, p. 38–43, 2010.
VAŇÁČOVÁ, Š.; RASOLOSON, D.; RÁZGA, J.; et al. Iron-induced changes in pyruvate metabolism of Tritrichomonas foetus and involvement of iron in expression of hydrogenosomal proteins. **Microbiology**, v. 147, n. 1, p. 53–62, 2001.
VERGINE, P.; SOUSA, F.; LOPES, M.; et al. Synthetic soft drink wastewater suitability for the production of volatile fatty acids. **Process Biochemistry**, v. 50, n. 8, p. 1308–1312, 2015. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.procbio.2015.04.007>. .
WANG, X.; MONIS, P. T.; SAINT, C. P.; JIN, B. Biochemical kinetics of fermentative hydrogen production by Clostridium butyricum W5. **International Journal of Hydrogen Energy**, v. 34, n. 2, p. 791–798, 2009. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2008.11.023>. .
WICKHAM, R.; XIE, S.; GALWAY, B.; BUSTAMANTE, H.; LONG NGHIEM. Anaerobic digestion of soft drink beverage waste and sewage sludge. **Bioresource Technology**, 2018.
WIMONSONG, P.; NITISORAVUT, R.; LLORCA, J. Application of Fe – Zn – Mg – Al – O hydrotalcites supported Au as active nano-catalyst for fermentative hydrogen production. **CHEMICAL ENGINEERING JOURNAL**, v. 253, p. 148–154, 2014. Elsevier B.V. Disponível em: <http://dx.doi.org/10.1016/j.cej.2014.05.047>. .
WU, H.; WANG, C.; CHEN, P.; HE, A. Y.; et al. Effects of pH and ferrous iron on the coproduction of butanol and hydrogen by Clostridium beijerinckii IB4. **International Journal of Hydrogen Energy**, v. 42, n. 10, p. 6547–6555, 2017. Elsevier Ltd.
WU, H.; WANG, C.; CHEN, P.; HE, A.; et al. ScienceDirect Effects of pH and ferrous iron on the coproduction of butanol and hydrogen by Clostridium beijerinckii. **International Journal of Hydrogen Energy**, p. 1–9, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2017.02.094>. .
XIAO, X.; HOU, Y.; LIU, YANG; et al. Talanta Classification and analysis of corn steep liquor by UPLC / Q-TOF MS and HPLC. **Talanta**, v. 107, p. 344–348, 2013. Elsevier. Disponível em: <http://dx.doi.org/10.1016/j.talanta.2013.01.044>. .
YANG, G.; WANG, J. Various additives for improving dark fermentative hydrogen production : A review. **Renewable and Sustainable Energy Reviews**, v. 95, n. July, p. 130–146, 2018a. Elsevier Ltd. Disponível em: <https://doi.org/10.1016/j.rser.2018.07.029>. .
YANG, G.; WANG, J. Improving mechanisms of biohydrogen production from grass using zero-valent iron nanoparticles. **Bioresource Technology**, v. 266, n. May, p. 413–420, 2018b. Elsevier.
YANG, T.; RAO, Z.; ZHANG, X.; XU, M. Effects of corn steep liquor on production of 2 , 3-butanediol and acetoin by Bacillus subtilis. **Process Biochemistry**, v. 70, p. 20–28, 2018. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.procbio.2013.07.027>. .
YE, X.; ZHANG, X.; MORGENROTH, E.; FINNERAN, K. T. Anthrahydroquinone-2 , 6-disulfonate increases the rate of hydrogen production during Clostridium beijerinckii fermentation with glucose , xylose , and cellobiose. **International Journal of Hydrogen Energy**, v. 37, n. 16, p. 11701–11709, 2012. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2012.05.018>. .
YIN, Y.; WANG, J. ScienceDirect Isolation and characterization of a novel strain

Clostridium butyricum INET1 for fermentative hydrogen production. **International Journal of Hydrogen Energy**, v. 42, n. 17, p. 12173–12180, 2017. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.ijhydene.2017.02.083>. .
ZAMPOL, C.; BERNADETE, M.; VARESCHE, A.; EDSON, S. Sequential fermentative and phototrophic system for hydrogen production : An approach for Brazilian alcohol distillery wastewater. **International Journal of Hydrogen Energy**, v. 40, n. 31, p. 9642–9655, 2015. Elsevier Ltd.
ZAMPOL, C.; PERNA, V.; ETCHEBEHERE, C.; VARESCHE, M. Sugarcane vinasse as substrate for fermentative hydrogen production : The effects of temperature and substrate concentration. **International jorurnal of hydrogen energy**, v. 9, p. 6407–6418, 2014.
ZHANG, W.; XIE, Q.; ROUSE, J. D.; QIAO, S.; FURUKAWA, K. Treatment of high-strength corn steep liquor using cultivated Polyvinyl alcohol gel beads in an anaerobic fluidized-bed reactor. **Journal of Bioscience and Bioengineering**, v. 107, n. 1, p. 49–53, 2009. The Society for Biotechnology, Japan.
ZHANG, Y.; SHEN, J. Enhancement effect of gold nanoparticles on biohydrogen production from artificial wastewater. , v. 32, p. 17–23, 2007.
ZHAO, W.; ZHANG, Y.; DU, B.; et al. Bioresource Technology Enhancement effect of silver nanoparticles on fermentative biohydrogen production using mixed bacteria. **Bioresource Technology**, v. 142, p. 240–245, 2013. Elsevier Ltd. Disponível em: <http://dx.doi.org/10.1016/j.biortech.2013.05.042>. .
ZHAO, X.; XING, D.; FU, N.; LIU, B.; REN, N. Bioresource Technology Hydrogen production by the newly isolated Clostridium beijerinckii RZF-1108. , v. 102, p. 8432–8436, 2011.
ZHAO, X.; XING, D.; LIU, B.; et al. The effects of metal ions and L -cysteine on hyd A gene expression and hydrogen production by Clostridium beijerinckii. , v. 7, 2012.