

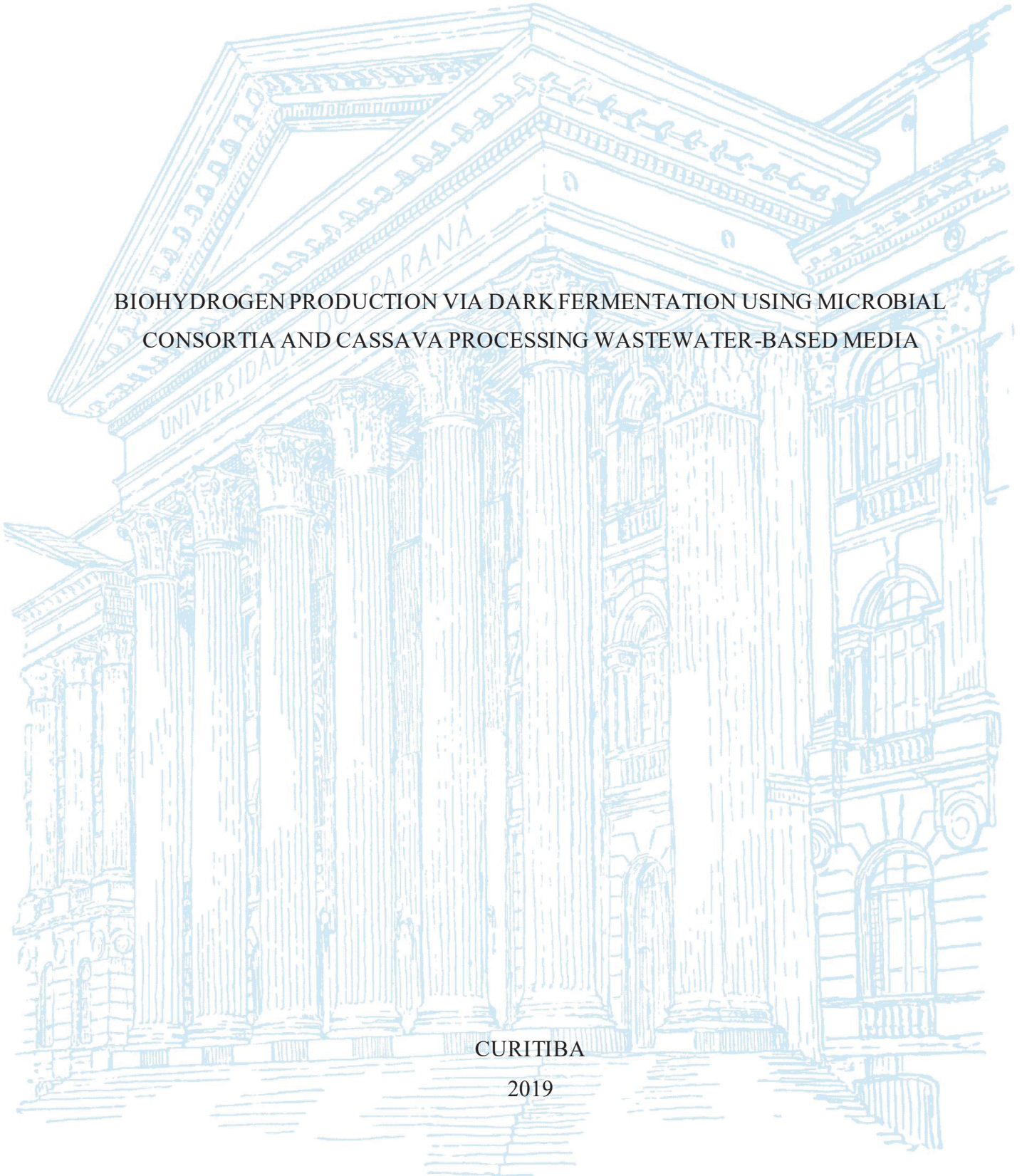
UNIVERSIDADE FEDERAL DO PARANÁ

WALTER JOSÉ MARTÍNEZ BURGOS

BIOHYDROGEN PRODUCTION VIA DARK FERMENTATION USING MICROBIAL
CONSORTIA AND CASSAVA PROCESSING WASTEWATER-BASED MEDIA

CURITIBA

2019



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CONSORTIA AND CASSAVA PROCESSING WASTEWATER-BASED MEDIA.

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Coorientador: Prof. Dr. Eduardo Bittencourt Sydney
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“Não existem ideias más, sem erro não há crescimento” **Albert Einstein**

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RESUMO

Na transformação das matérias primas em produtos com valor agregado grandes quantidades de efluentes com composição variável e complexa são gerados, como é o caso da manipueira e da milhocina. Diferentes pesquisas têm mostrado que os efluentes citados têm sido usado como substrato na produção de diferentes metabolitos, contudo uma das áreas que mais promete é a produção de bioenergia. O Objetivo deste trabalho foi produzir bioH₂ usando efluentes agroindustriais e diferentes consórcios microbianos. Na primeira etapa deste estudo foram isolados e identificados a traves de técnicas moleculares cinco consórcios microbianos de diferentes ambientes brasileiros, também foi selecionada a melhor fonte de nitrogênio (estrato de levedura). Na segunda etapa as variáveis C/N, % inóculo, pH, T (°C) foram otimizadas em cada consorcio e se obteve um modelo matemático para cada consorcio. Ressalta-se que a pesquisa foi desenvolvida usando manipueira hidrolisada e esterilizada, manipueira bruta e manipueira esterilizada, a otimização foi feita com esta última. Na terceira etapa, foram avaliadas as condições ótimas de cada consorcio usando os três substratos, a produção de hidrogênio e de ácidos orgânicos foi monitorada usando cromatografia gasosa e HPLC, respectivamente. Finalmente, os consórcios com os melhores rendimentos (*Vir* and *Gal*) foram avaliados usando unicamente efluentes (manipueira e milhocina), além disso o processo foi escalonado até 5L.

PALAVRAS-CHAVES: Biohidrogênio. Manipueira. Consórcios microbianos. Otimização. Modelos matemáticos, Milhocina. Cinética.

ABSTRACT

In the transformation of raw materials into value-added products, large amounts of effluents with variable and complex composition are generated, as in the case of cassava processing wastewater (CPW) and corn steep liquor (CSL). Different researches have shown that the mentioned effluents have been used as substrate in the production of different metabolites, however one of the areas that most promises are the bioenergy production. The objective of this work was to produce bioH₂ using agro-industrial effluents and different microbial consortia. In the first stage of this study were isolated and identified through molecular techniques five microbial consortia from different Brazilian environments, and was also selected the best source of nitrogen (yeast stratum). In the second stage the variables C / N, inoculum %, pH, T (°C) were optimized in each consortium and a mathematical model was obtained for each consortium. It is noteworthy that the research was developed using hydrolyzed and sterilized CPW, crude CPW and sterile CPW and the optimization was made with the latter. In the third stage, the optimum conditions of each consortium were evaluated using the three substrates, and hydrogen and organic acids production was monitored using gas chromatography and HPLC, respectively. Finally, the best yielding consortia (*Vir* and *Gal*) were evaluated using only effluents (CPW and CSL), and the process was scaled up to 5L.

KEYWORDS: Biohydrogen. Cassava processing wastewater. Microbial consortia, Optimization, Mathematical models, Corn steep liquor, Kinetics.

FIGURES LIST

FIGURE 1 - Simplified process of production : (A) Slaughterhouse process for animals and effluents generated, adapted (SCARASSATI et al., 2003) ; (B) Milk processing, effluent generation and whey, adapted (FEAM and FIEMG, 2013); (C) Extraction palm oil and POME generation, adapted (AHMED et al.,2015) Fresh fruit bunches (FFB), Empty fruit bunches (EFB); (D) Process of starch or cassava flour and generation of CPW, adapted (SARRIA NÚÑEZ, 2011;TRAN et al., 2015); (E) Process of alcohol .production and SV eneration, adapted (CHRISTOFOLETTI et al., 2013b; e Rodrigues and Hu, 2017). The operations in the box with oblique lines require energy.	22
FIGURE 2 - Variability of the physicochemical composition of effluents. A) COD; B) BOD; C) N; D) P; E) pH.....	25
FIGURE 3 - Areas most impacted by effluents in Brazil	27
FIGURE 4 - Different bio-products produced from agro-industrial effluents.	31
FIGURE 5 - BioH ₂ produced from the different sources of nitrogen.....	46
FIGURE 6 - Rarefaction curve for the bacterial community consortia <i>Lod</i> , <i>Vac</i> , <i>Esg</i>	47
FIGURE 7 - Microbial composition for the Vac, Esg and Lod consortia. Genetic analysis based on the V4 region of the 16s rDNA gene.....	48
FIGURE 8 - Projection of response surfaces for bioH ₂ production by Vac consortium as a .	49
FIGURE 9 - Projection of response surfaces for bioH ₂ production by Esg consortium as a function of pH (4.5-6.5), T °C (32-42°C), Inoculum ratio (10%-20%), and C/N ratio (3-5); coded values.....	51
FIGURE 10 - Projection of response surfaces for bioH ₂ production by Lod consortium as a function of pH (4.5-6.5), T °C (32-42°C), Inoculum ratio (10%-20%), and C/N ratio (2-5); coded values.....	52
FIGURE 11 - Microbial growth profile (A) <i>Lod</i> consortium, (B) <i>Esg</i> consortium, (C) <i>Vac</i> consortium.	55
FIGURE 12 - Kinetic profile of bioH ₂ production with <i>Vac</i> , <i>Esg</i> and <i>Lod</i> consortia.	56
FIGURE 13 - Cumulative volume of bioH ₂ produced by different consortia and culture media.	57
FIGURE 14 - Intermediate metabolic products (Acetic acid, butiric acid, succinic acid, lactic acid and ethanol) generated by the consortia <i>Vac</i> , <i>Esg</i> and <i>Lod</i> in media H, M and B.....	59
FIGURE 15 - BioH ₂ produced from the different sources of nitrogen.....	70

FIGURE 16 - Rarefaction curve for the bacterial community <i>Gal</i> and <i>Vir</i> consortia (times 0, 8, 16, 24 and 48h).	71
FIGURE 17 - Microbial composition for the <i>Gal</i> and <i>Vir</i> consortia. Genetic analysis based on the V4 region of the 16s rDNA gene.	72
FIGURE 18 - Optimized variable response surfaces. (A) <i>Vir</i> Consortium, (B) <i>Gal</i> Consortium.	76
FIGURE 19 - Real values vs. predicted values for the models, <i>Vir</i> (A) and <i>Gal</i> (B).	78
FIGURE 20 - Microbial growth profile (A) <i>Vir</i> consortium, (B) <i>Gal</i> consortium.	79
FIGURE 21 - Kinetic profile of bioH ₂ production with <i>Vir</i> and <i>Gal</i> consortia	81
FIGURE 22 - . Kinetics of substrate consumption and generation of some metabolites. Substrate (A) maltose and glucose-G), (B) Acetic acid, (C) Butyric acid, (D) Lactic acid, (E) Ethanol, (F) pH.	84
FIGURE 23 - Kinetics of the relative abundance of the species of each consortium (HS	86
FIGURE 24 - Bioreactor system for bioH ₂ production.	93
FIGURE 25 – (A) Standardized effects for <i>Gal</i> consortium ($\alpha:0.05$) (B) Standardized effects for <i>Vir</i> consortium ($\alpha:0.05$).	95
FIGURE 26 - Contour and response surfaces for <i>Vir</i> and <i>Gal</i> consortia. (A) and (B): <i>Vir</i> Consortium; (C) and (D): Consortium <i>Gal</i>	97
FIGURE 27 - Adjustment of the residuals to a normal distribution (<i>Vir</i> consortium: A, <i>Gal</i> consortium: B); Residual dispersion diagrams (<i>Vir</i> consortium: C, <i>Gal</i> consortium: D) and actual values vs. predicted values for the models <i>Vir</i> (E) and <i>Gal</i> (F).	100
FIGURE 28 - Cumulative production of bioH ₂	101
FIGURE 29 - (A) Carbohydrate consumption; (B) Production of organic acids.	102
FIGURE 30 - Volume of bioH ₂ generated at different scales.....	105

TABLES LIST

TABLE 1 - Estimated quantities of agro-industrial effluents generated in Brazil in 2017.	23
TABLE 2 - Potential environmental impacts generated by agroindustrial effluents.....	28
TABLE 3 - Bioreactors used for wastewater treatment and biogas production (H ₂ and CH ₄).33	
TABLE 4 - Amount of energy contained in effluents produced in Brazil.	36
TABLE 5 - Origins of bacterial consortia in Brazilian environments.	40
TABLE 6 - BB design matrix for the consortia <i>Vac</i> , <i>Esg</i> and <i>Lod</i> , experimental and predicted results for bioH ₂ production.....	43
TABLE 7 - Physical-chemical composition of CPW.....	45
TABLE 8 - Characteristics and parameters of mathematical models for the production of bioH ₂ by <i>Lod</i> , <i>Esg</i> and <i>Vac</i>	53
TABLE 9 - Comparison between the volumes of bioH ₂ produced in the optimum experimental conditions and the models	54
TABLE 10 - Origins of bacterial consortia in Brazilian environments.	64
Table 11 - Physicochemical characteristics of CPW	65
TABLE 12 - Independent variable intervals.....	74
TABLE 13 - Characteristics and parameters of <i>Vir</i> and <i>Gal</i> models.	77
TABLE 14 - Kinetic parameters BioH ₂ production.	80
TABLE 15 - Parameters used in scale-up.....	93

LIST OF ABBREVIATIONS OR ACRONYMS

ANOVA: Analysis of variance

BB: Box-Behnken

CCD: Composite central design

COD: Chemical oxygen demand

CPW: Cassava Processing Wastewater

CSL: Corn steep liquor

EFB: Empty fruit bunches

EMP: Milk processing effluents

Esg: Lodo de esgoto

FFB: Fresh fruit bunches

Gal: Esterco de galinha

GDP: Gross domestic product

HPLC: High-Performance Liquid Chromatography

Lod: Lodo de manipueira

PB: Placket-Burman

POME: Palm oil mill effluent

Pt: Purge time

SH: Slaughterhouse effluents

SV: Sugarcane vinasse

Vac: Esterco de gado

Vir: Lodo de vinhaça

SUMMARY

1.	GENERAL INTRODUCTION	16
2.	CHAPTER I (AGRO-INDUSTRIAL WASTEWATER AS POTENTIAL SOURCE OF NEW BIOPRODUCTS: CHARACTERISTICS, ENVIRONMENTAL IMPACTS AND BIOPROCESSING).....	18
2.1	ABSTRACT.....	18
2.2	INTRODUCTION.....	19
2.3	AGRIBUSINESS, RAW MATERIAL PROCESSING AND EFFLUENT GENERATION.....	20
2.4	CHARACTERISTICS OF AGRO-INDUSTRIAL EFFLUENTS.....	24
2.5	ENVIRONMENTAL IMPACTS OF AGRO-INDUSTRIAL EFFLUENTS ..	26
2.6	VALUE-ADDED PRODUCTS OF INDUSTRIAL INTEREST THAT COULD BE PRODUCED WITH EFFLUENTS.....	30
2.7	CONCLUSION AND PERSPECTIVES	36
3.	CHAPTER II (THE EFFECT OF HYDROLYSIS AND STERILIZATION IN BIOHYDROGEN PRODUCTION FROM CASSAVA PROCESSING WASTEWATER MEDIUM USING ANAEROBIC BACTERIAL CONSORTIA)	37
3.1	ABSTRACT.....	37
3.2	INTRODUCTION.....	38
3.3	MATERIALS AND METHODS.....	39
3.3.1	Consortia sampling	39
3.3.2	Molecular identification of consortia	40
3.3.2.1	Illumina high-throughput sequencing.....	40
3.3.2.2	Bioinformatics and data analysis.....	40
3.3.3	Experimental design and statistical analyses.....	41
3.3.3.1	Selection of variables	41
	3.3.3.1.1 Nitrogen source	41
	3.3.3.1.2 Optimization	42
3.3.4	Microbial kinetic profile	43

3.3.5	Kinetic profile of bioH ₂ production.....	44
3.3.6	Analyses of organic compounds.....	44
3.4	RESULTS AND DISCUSSION.....	44
3.4.1	Selection of the nitrogen source	45
3.4.2	Molecular identification of consortia	47
3.4.3	Selection other variables and optimization of bioH ₂ production.....	48
3.4.4	<i>Vac</i> , <i>Esg</i> and <i>Lod</i> optimizations	49
3.4.5	Mathematical Models	52
3.4.6	Analysis of bioH ₂ production under optimum conditions.....	54
3.5	CONCLUSION	59
4.	CHAPTER III (BIOHYDROGEN PRODUCTION IN CASSAVA PROCESSING WASTEWATER USING MICROBIAL CONSORTIA: KINETIC PARAMETERS AND OPTIMIZATION)	61
4.1	ABSTRACT.....	61
4.2	INTRODUCTION.....	62
4.3	MATERIALS AND METHODS.....	64
4.3.1	Consortia sampling	64
4.3.2	Experimental design and statistical analyses.....	64
4.3.3	Selection of the nitrogen source	65
4.3.4	Optimization	66
4.3.5	Microbial kinetic profile	66
4.3.6	Kinetic profile of bioH ₂ production.....	67
4.3.7	Kinetic profile of organic compounds production.....	67
4.3.8	Kinetic metagenomic profile	68
4.3.8.1	Molecular identification of consortia	68
4.3.8.2	Illumina high-throughput sequencing.....	68
4.3.8.3	Bioinformatics and data analysis.....	69
4.4	RESULTS AND DISCUSSION.....	69
4.4.1	Selection of the nitrogen source	69
4.4.2	Molecular identification of <i>Gal</i> and <i>Vir</i> consortia	71
4.4.3	Optimization of bioH ₂ production.....	73
4.4.4	Mathematical models.....	76

4.5	CONCLUSION	86
5.	CHAPTER IV (DEVELOPMENT OF A LOW-COST MEDIUM TO BIOH₂ PRODUCTION FROM AGROINDUSTRIAL EFFLUENTS AND SCALE-UP OF THE PROCESS)	88
5.1	ABSTRACT	88
5.2	INTRODUCTION.....	88
5.3	MATERIALS AND METHODS	90
5.3.1	Consortia and Culture Conditions	90
5.3.2	Agroindustrial effluents origin and characterization	91
5.3.3	Placket-Burman design.....	91
5.3.4	Optimization	91
5.3.5	Kinetic profile of bioH ₂ production.....	92
5.3.6	Analyses of organic compounds.....	92
5.3.7	Fermentation Scale-Up	92
5.4	RESULTS AND DISCUSSION.....	94
5.4.1	Physicochemical characterization	94
5.4.2	Optimization	96
5.4.3	Mathematical Models	98
5.4.4	Evaluation of bioH ₂ production under optimum conditions.....	101
5.5	CONCLUSION	106
6.	OVERALL CONCLUSION	107
7.	REFERENCES	108
8.	ANNEXES.....	147

1. GENERAL INTRODUCTION

Currently, around the world, there is a growing interest in searching for renewable sources with little or no impact on the environment, once approximately 87% of the energy consumed on the planet comes from burning of some kind of fossil fuel (coal, gas, oil) (PREETHI et al., 2019). This practice has been questioned by the negative effects that it causes on the environment and health of population (ABIÁN et al., 2019) besides these are resources that are running out.

Hydrogen is envisioned as one of the main energy sources of the future (MARTINEZ-BURGOS et al., 2019), as it has the characteristics of an ideal fuel: (i) it can be obtained in a renewable way, (ii) generates high amount of energy (iii) and its combustion does not generate greenhouse gases (only water). While the generation of energy from other fuels releases, considerable amounts of polluting gases such as CO₂ into the atmosphere. For example, the specific release of CO₂ to obtain 1MJ from methanol, ethanol, and gasoline is 68.44 g, 70.99 g, 73.95 g, respectively.

However, 95% of the hydrogen that is consumed around the world (500 billion m³) (KHAN et al., 2017) comes from fossil fuels, and only 5% come from renewable sources, from the electrolysis of the water molecule.

Hydrogen is the simplest element in the periodic table and one of the most abundant on the planet, approximately 75% of the visible matter contains H₂. However, hydrogen is always linked with other elements forming compounds such as carbohydrates, lipids, proteins, H₂O among others. For which different technologies and processes have been created to be able to isolate H₂ from other elements. However, most of these technologies have in common high-energy consumption.

A promising alternative is the biological production of hydrogen using microbial consortia (dark fermentation) and industrial and agro-industrial effluents as substrates, which are generated in high quantities and with high levels of pollution. For example, cassava processing wastewater (CPW), corn steep liquor (CSL) and sugarcane vinasse (SV) are generated approximately to a 300-600 L of CPW / ton cassava (DE CARVALHO et al., 2018; SILVA et al., 2014), 200-500 kg of CSL / ton corn and 15L of SV / L ethanol (SYDNEY et al., 2019), respectively. This hydrogen production pathway has marked advantages, for example it reduces the organic effluent load up to 90%,

removes much of the nitrogen and phosphorus from the effluents (elements responsible for eutrophication in water bodies) and the difference in traditional processes (steam reforming, partial oxidation, autothermal reforming) in dark fermentation bioreactors can operate at ambient temperatures (25-40 °C) and low pressures.

The main applications of hydrogen are in oil refining, the production of fertilizers and chemical compounds. Currently, the energy sector is gaining a lot, in fact until April 2018, 8000 cars already used H₂ fuel in electrochemical cells (NAGARAJAN et al., 2019).

This work focused on bioH₂ production using agro-industrial effluents as substrate and different microbial consortia. The research evaluated different nitrogen sources, optimized some variables, determined some kinetic parameters and bioH₂ productivity up to 5L.

2. CHAPTER I (Agro-industrial wastewater as potential source of new bioproducts: characteristics, environmental impacts and bioprocessing)

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2.1 Abstract

Nowadays, world's environmental challenges are oriented to the use of agroindustrial wastewater in the generation of new bioproducts and energy with the consequent reduction in pollution. Brazil is the second largest producer in the agroindustrial sector around the world. The volumes of effluents generated by this sector are extremely elevated, with high levels of pollution, chemical and biochemical oxygen demand, toxic compounds and pathogenic microorganisms. The characteristics of wastewater are variable and can be used mainly in three areas: generation of metabolites of industrial interest, power generation and fertirrigation processes. This review describes where the industrial processes generate agro-effluents in the production of ethanol, cassava starch and palm oil, by the dairy industry and in slaughterhouses, the physicochemical characteristics of these effluents, their impacts and biotechnological applications explored by the scientific community aiming at the valorization of these byproducts.

Keywords: Bioprocess; cassava; vinasse; dairy processing; POME; slaughter.

2.2 Introduction

Environment preservation of the is one of the challenges for mankind, mainly because it is affected by many human activities developed to satisfy their needs. Mainly because the environment is negatively impacted by the activities that man develops to meet his nutritional, energy, transportation needs, etc. Among these, it is important to highlight agro-industrial businesses, which affect the three axes of the environment (water, soil and air), due to the high amounts of effluents that can be generated and its high level of pollution

In Brazil, the agro-industrial sector is economically important, responsible for approximately 25% of gross domestic product (GDP) (MAPA, 2019a). The main products are those derived from sugarcane (alcohol and sugar), cassava (flour and starch), cereals (maize and rice), oilseeds (soybean), and animal slaughter (cattle, pigs, and poultry) (COSTA et al., 2013). Despite its economic importance, agroindustry generates enormous volumes of wastewater rich in organic matter that need to be properly addressed. Alternatively, by the simple physicochemical and biological treatment of these effluents, it is possible to exploit these industrial wastes in the development of innovative processes for the generation of new bioproducts, promoting effluent valorization and developing the circular economy. In recent years effluents from the production of ethanol sugarcane vinasse (SV), cassava processing wastewater (CPW), slaughterhouses (SH), milk processing (EMP), whey (mainly in small industries) and extraction of palm oil (POME), have been extensively studied to produce metabolites of industrial interest, such as organic acids, alcohols, proteins, microbial biomass, methane, biohydrogen, biobutanol, etc. In addition, these effluents have also been applied to obtain bioenergy, an attractive option, since it reduces up to 92% of the organic load and allows the use of energy generated within the production company itself (circular economy). In Brazil, for example, the energy contained in these effluents is about 10% of the electrical energy consumed in the year 2016. This work summarizes the ways of recovering wastewater generated in the processing of sugarcane to obtain ethanol, starch from cassava, oil from palm, whey from dairy products and the slaughter of animals. We also describe the characteristics, biotechnological applications and impacts of such effluents.

2.3 Agribusiness, raw material processing and effluent generation

Agribusiness can be defined as a set of economic activities including production, processing or industrialization and commercialization of agricultural and forestry products (DA SILVA et al., 2009), either for food or for non-alimentary purposes. These activities require large amounts of energy and water, generating expressive volumes of solid materials and wastewater with important polluting characteristics (SAVAL, 2012). Agro-industrial effluents may not be useful in the process that has generated them but have the potential to be used or processed to generate different products with significant economic value.

However, the processing of these raw materials generates huge volumes of effluents with high eutrophic and polluting potential, due to the fact that they have to be treated before being disposed into the environment (DE-BASHAN and BASHAN, 2004). According to CHRISTOFOLETTI et al. (2013); GAJENDRAGADKAR and GOGATE (2016) waste management and disposal in recent years have received special attention, as large amounts of waste are generated each year, which is a concern around world.

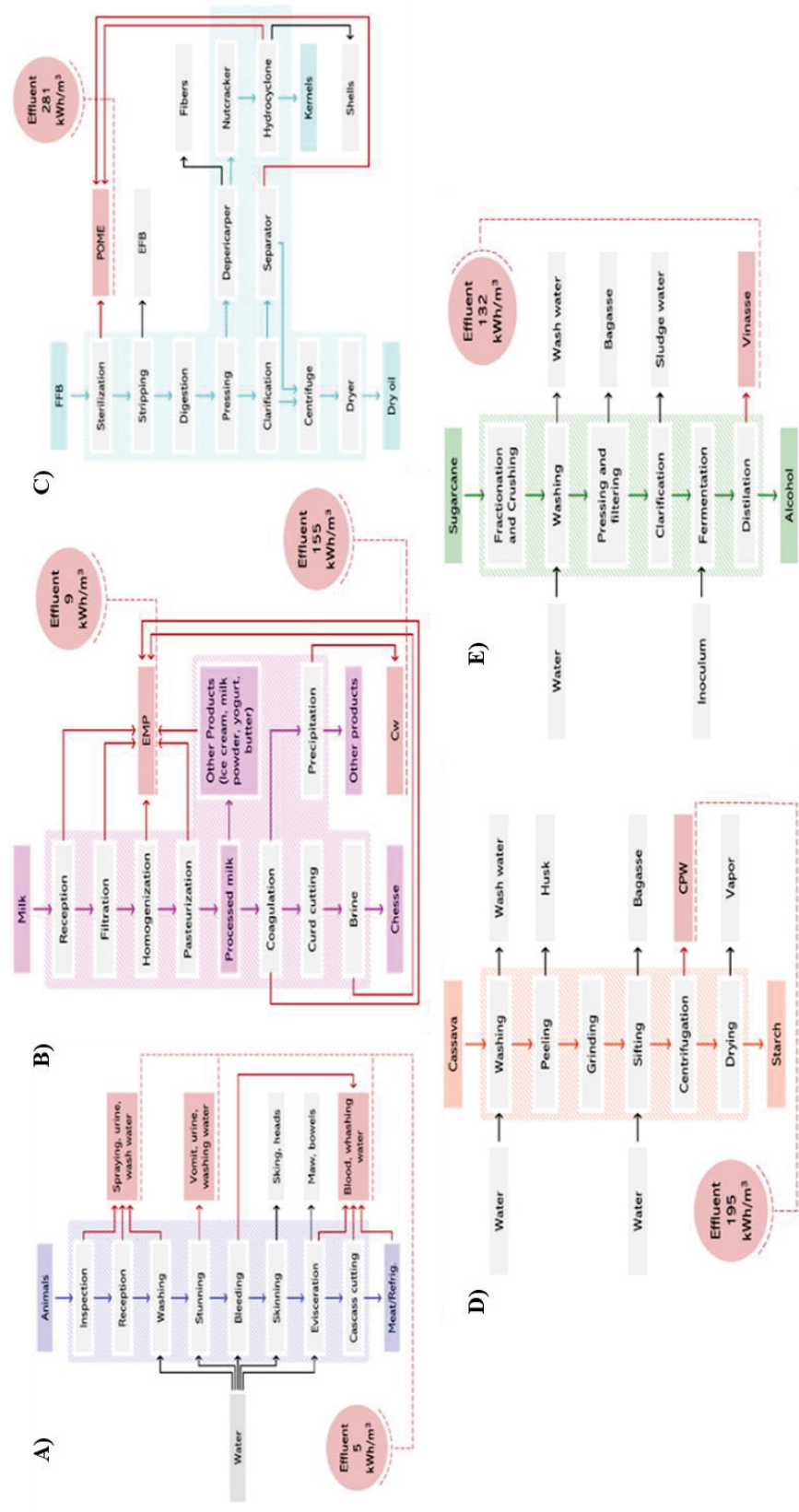
Moreover, these effluents are different and have a complex composition as will be described below, whereby they may impact the ecosystems and their species in different ways (CHRISTOFOLETTI, et al., 2013). In Brazil, the main agro-industrial processes that generate effluents are cassava, sugar cane, milk, palm oil and animal slaughter processing. As shown in FIGURE 1, effluents can be generated in different steps or a single step of the process. Thus, in animal slaughtering and milk processing effluents are generated practically during the whole process (FIGURES 1A and 1B). Whey, which is mainly associated with cheese production, can be considered an effluent or not. Besides, POME, an effluent produced by the processing of African palm seeds, is specifically generated in three steps: sterilization and separation, and hydro cyclone (FIGURE 1C) (AHMED et al., 2015).

However, in other agro-industrial processes such as cassava processing for the production of starch and sugar cane for alcohol, the CPW and VW effluents are generated specifically in the centrifugation and distillation steps, respectively (FIGURES 1D e 1E) (CHRISTOFOLETTI et al., 2013; MORAES et al., 2015; SARRIA NÚÑEZ, 2011;

TRAN et al., 2015) . It is observed that these effluents are a significant source of energy. For example, the effluents with the highest energy content by volume are POME, CPW, cheese whey (Cw), and sugarcane vinasse with 281, 195, 155 and 132 kWh/m³, respectively. Although slaughterhouse (SH) and milk processing (EMP) effluents contain the lowest energy content, their use could be justified given the high volumes that are generated.

Brazil is one of the main producers of products from the agricultural sector, through which it directly generates large volumes of effluents. For example, this country is the largest producer of sugar cane in the world, the fifth largest producer of cassava, and the fourth-largest producer of pig meat (GERBER et al., 2017). Also produces significant amounts of beef and chicken (AZIZ et al., 2019), as well as milk and dairy products.

FIGURE 1 - Simplified process of production : (A) Slaughterhouse process for animals and effluents generated, adapted (SCARASSATI et al., 2003) ; (B) Milk processing, effluent generation and whey, adapted (FEAM and FIEMG, 2013); (C) Extraction palm oil and POME generation, adapted (AHMED et al., 2015) Fresh fruit bunches (FFB), Empty fruit bunches (EFB), Process of starch or cassava flour and generation of CPW, adapted (SARRIA NÚÑEZ, 2011; TRAN et al., 2015); (D) Process of alcohol .production and SV eneration, adapted (CHRISTOFOLETTI et al., 2013b; e Rodrigues and Hu, 2017). The operations in the box with oblique lines require energy.



** Discontinue lines indicated effluent that can be used in the generation of bioenergy or other types of metabolites

Thus, 219 million tons of cassava were produced worldwide in 2017, and Brazil produced approximately 8.6% the same year (FAOSTAT, 2019). According to MADEIRA et al. (2017), in this country, 60% of the cassava produced is processed. In other words, in 2017 approximately 6.8 million m³ of CPW were generated. On the other hand, between 2017 and 2018 approximately 620.1 million tons of sugarcane were produced, which were processed into 33 million m³ of alcohol and 29.1 million tons of sugar (MAPA, 2019b), and generated more than 497 million m³ of SV. Other raw materials and their generated effluents are summarized in TABLE 1.

TABLE 1 - Estimated quantities of agro-industrial effluents generated in Brazil in 2017.

Raw material processed	Effluent	Effluent generation ratio	Generation m ³
18.876.470 million ton	CPW	600L CPW/1 ton cassava (DE CARVALHO et al. 2018)	6.795.529
33.2 million m ³	Vinasse	15 L Vinasse /L ethanol (SYDNEY et al., 2019)	497.333.160
321.398 ton SNA (2016)	POME	3.75 ton POME/1ton Crude palm oil (CHENG et al., 2018)	1.285.592
35 million m ³ produced (24 million m ³ processed)	EMP	5.1 L effluent/ L processed milk (VOURCH et al., 2008)	122.400.000
7.5 million m ³ cheese production	Whey	0.9 m ³ whey/ 1m ³ of milk processed (GONZÁLEZ SISO 1996;Parra, 2009)	6.500.000
5372 million of slaughtered chickens (2017) (MAPA, 2019c)	Slaughterhouse effluents	0.037 m ³ /slaughtered chicken (SCARASSATI et al., 2003)	236.362.158
36.7 million of slaughtered pigs (2017) (MAPA, 2019c)		1.2 m ³ /slaughtered pig (SCARASSATI et al., 2003)	
23.2 million of slaughtered cattles (2017) (MAPA, 2019c)		2.5 m ³ /slaughtered cattle (SCARASSATI et al., 2003)	

It is observed that the agro-industrial activities that generate more effluents are ethanol production, followed by animal slaughter and milk processing. In the world, there

is a growing concern about the exorbitant amounts of waste being produced by man. Thus, one of the industries that are most concerned about the amount of effluents generated is the slaughtering of animals. It is estimated that each year this industry generates approximately 4.96 billion m³ of effluents (AZIZ et al., 2019; MBURU et al., 2019). It is estimated that China and the United States lead meat producing generate approximately 1.3 and 0.7 billion m³ of wastewater each year, respectively. Another type of industry questioned is ethanol producers. According to SYDNEY et al. (2019), in 2018, approximately 1.6 billion m³ SV was generated from sugarcane ethanol production in the world. In the case of POME, it is estimated that every year the major producers Indonesia and Malaysia (85% of the producers of palm oil) generate on average 190 million tons of POME (CHENG et al., 2018; PALTSEVA et al., 2016).

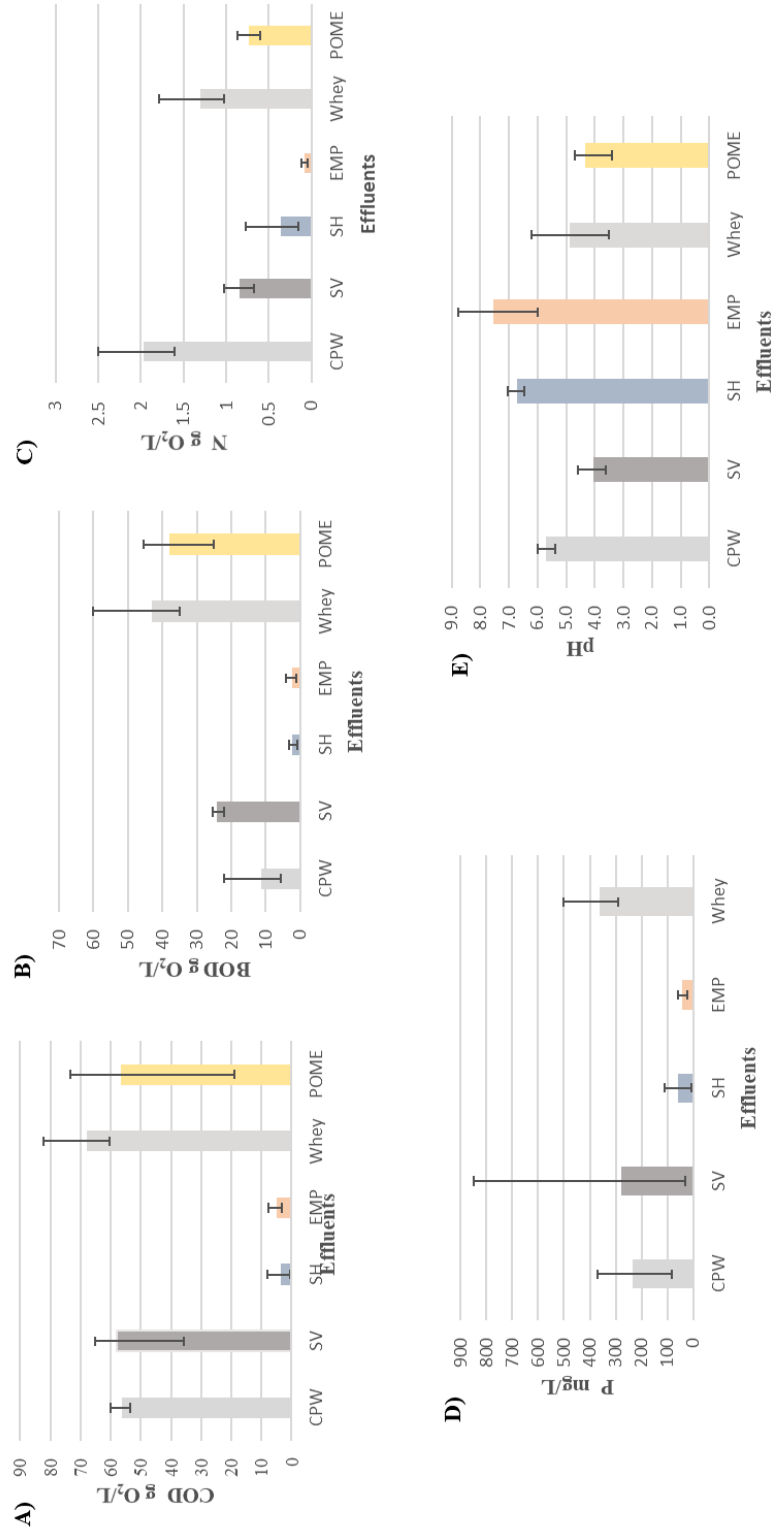
2.4 Characteristics of agro-industrial effluents

The physicochemical characteristics effluents are highly different, even among the same effluent, as illustrated in FIGURE 2. The variability can be attributed to the type of process and technology and characteristics of the raw material: for plants, the variety, nutrition, season, etc., and for animals the breed, feed, and environmental conditions (OKUNADE and ADEKALU, 2014; AZIZ et al., 2019).

The effluents CPW, VW, whey and POME contain the largest organic loads (COD), on average 55 g O₂/L (FIGURE 2A). This is explained by the fact that these effluents contain high levels of carbohydrates and proteins. Besides, these effluents also contain significant concentrations of other nutrients such as nitrogen (FIGURE 2C) and phosphorus (FIGURE 2D). Although SH and EMP have the lowest organic loads, these are around 5g O₂/ L, still considered very high. Because environmental legislation has established that COD should not exceed 50 mg O₂/L, pH (5-9) and total nitrogen below 200 ppm (HAMEED et al., 2003), all these effluents need to be treated before being disposed into the environment.

The main carbon sources that the effluents contain are starch, maltose, and glucose in the case of CPW (CAPPELLETTI et al., 2011; MARTINEZ-BURGOS et al., 2019). Whey and EMP mainly contain lactose as carbon sources (approximately 50 g / L) (Akhlaghi et al., 2017), whey also contains significant concentrations of lipids.

FIGURE 2 - Variability of the physicochemical composition of effluents: (A) COD; (B) BOD; (C) N; (D) P; (E) pH.



SOURCE:

(DAMASCENO et al., 2003); NITSCHKE, 2003; NITSCHKE, 2006; MARÓSTICA and PASTORE, 2006; CAVALCANTE et al., 2008; ROBLES-GONZÁLEZ et al. 2012, NITAYAVARDHANA et al. 2013; AGUILAR et al., 2002; BUSTILLO-LECOMPTÉ et al., 2015; BUSTILLO-LECOMPTÉ and MEHRVAR, 2016; RAJAB et al., 2017; BHATIA et al., 2007; RUPANI et al., 2010; AHMED et al., 2015; NORFADILAH et al., 2016; SINGH et al., 2013; SIRIANUNTAPIBOON et al., 2005; TAWFIK et al., 2008; GELEGENIS et al., 2007; VENETSANEAS et al., 2009; SADDODUD et al., 2007; BOTELHO et al., 2009; SUN et al., 2012; CARDOSO, 2005).

In the case of POME, there is a low concentration of easily degradable sugars (NORFADILAH et al., 2016), however, it contains lignocellulosic material which can be hydrolyzed to provide carbohydrates (KHADAROO et al., 2019).

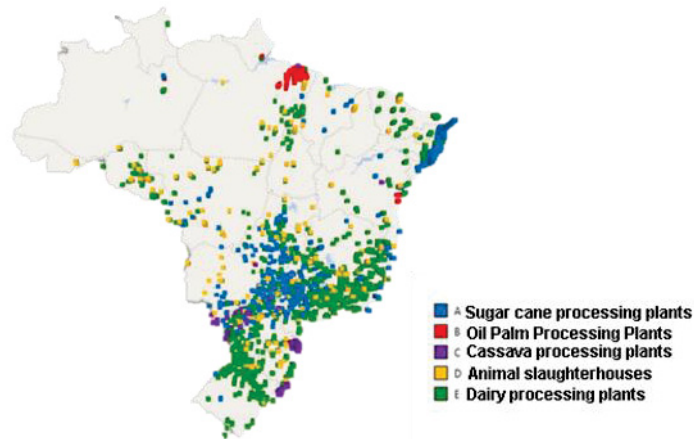
Besides, POME, whey, SH and EMP also contain high lipids (fats or oils) which can also be used as carbon sources. These compounds are also responsible for the high COD of effluents (VENETSANEAS et al., 2009; GELEGENIS et al., 2007; AHMED et al., 2015, NORFADILAH et al., 2016). Also, effluents are sources of micronutrients such as Ca, S, K and Mg (AZIZ et al., 2019; NITAYAVARDHANA et al., 2013; CAVALCANTE et al., 2008; NORFADILAH et al., 2016). In addition, it was observed that the most neutral pH effluents were SH and EMP and the most acidic VW and whey with pH around 4.5 (FIGURE 2E). Generally, the temperatures of these effluents are between 20 and 35 °C, except POME and VW leaving the process with temperatures around 85 °C (NORFADILAH et al., 2016; MORAES et al., 2015). Before being disposed, these effluents must be cooled.

2.5 Environmental impacts of agro-industrial effluents

Agro-industrial wastewaters have high concentrations of N and P and when discarded into bodies of water, they promote the growth of microorganisms, aquatic plants and microalgae. Eutrophication affects other species and completely destabilizes ecosystems, making the receiving waters unfit for other uses (SILVA et al., 2005). The São Paulo state, the main producer of sugar cane in Brazil, has serious problems of contamination and water shortage due to several factors: (i) large quantities are used in irrigation processes, (ii) big volumes are used in the production of ethanol, and (iii) water used in the industrial sector is highly polluted. FIGURE 3 shows that the most impacted regions in Brazil are: the south, southeast and midwest.

These effluents can also impact the soil when used in fertirrigation. The indiscriminate use of effluents such as CPW in this process increases the soil salt content (ABEGUNRIN et al., 2016). Fertirrigation with domestic sewage mainly affects alkalinity; the SV provides large amounts of chlorides and nitrates that can reach the groundwater through leaching processes (FUESS and GARCIA, 2014), and would trigger soil contamination by toxic ions and metals.

FIGURE 3 - Areas most impacted by effluents in Brazil



SOURCE: Agropalma (2019), Mapa (2019) and Novacana (2019).

Another risk associated with the use of wastewater in fertirrigation is the hydrophobicity that the soil can acquire, generated mainly by the organic matter and the microorganisms covering the surface (VOGELMANN et al., 2013). The organic overload can clog the pores of the soil, decrease the dissolved oxygen, and exchange the microbial population for an undesirable one (FUESS and GARCIA, 2014).

Another worrying impact of effluents is the generation of different greenhouse gases. The organic matter of the CPW can be biodegraded to CH₄ and CO₂, gases with potent greenhouse effect (AYU and ARYATI, 2010; SILVA et al., 2005), and which can be released into the atmosphere when effluents are improperly disposed of.

Effluents such as POME, EMP, VW and slaughterhouses also generate these gases. However, the latter can also generate other gases such as NO_x and SO_x, responsible for acid rain (DANESH et al., 2010; YANG et al., 2012). CARMO et al. (2013) found that sugarcane crops fertilized with VW had higher fluxes of both CO₂ and NO₂ with values of approximately 38,000 mg m⁻² day⁻¹ and 40 mg m⁻² day⁻¹ respectively. TABLE 2 summarizes other types of impacts associated with agroindustrial effluents.

TABLE 2 - Potential environmental impacts generated by agroindustrial effluents.

Type of Impacts	Effluents				References
	CPW	VW	Slaughterhouse effluent	POME	Whey
Presence of compounds with toxic nature	+cyanogenic glycosides (1)	Pb, Zn (7)	-	+(20, 21) Heavy metals	-
Changes in ecosystems	+(5)	+(7)	+(13)	+(20)	+(27)
Changes in soil physical and chemical properties	+(2)	+(8)	+(14)	+(22)	+(28)
Changes in soil microbial population	+(3)	+(8)	+(14)	-	+(29)
Eutrophication	+(4)	+(9)	+(15)	+(23)	+(30)
Water resource depletion	+(4)	+(10)	+(16)	+(24)	+(30)
Increasing the temperature of the receiving water bodies	-	+(11)	-	+(25)	-
Unpleasant odors	+(5)	+(9)	+(17)	+22	+(30)

RIVADENEYRA-DOMÍNGUEZ et al. (2013)¹ FUESS AND GARCIA (2014)⁷ AZMAN et al., (2016)²⁰ IGWE and ONYEGBADO (2007)²¹ KOBAYASHI et al. (2006)¹³ DE GIOANNIS et al. (2014)²⁷ ABEGUNRIN et al. (2016)² GERBER, M. D. et al. (2017) e DA ROCHA et al. (2012)¹⁴ ALI AMAT et al. (2015)²² GHIMIRE et al. (2017) FALCAO (2016)²⁹ FUESS and GARCIA (2014)³ DA SILVA et al. (2013)⁸ MARONE et al. (2017)²⁹ PAPONG et al. (2014) and SEN and SUTTAR (2012)⁴ CRISTOFOLETTI et al., (2013b)⁹ AGUILAR et al., (2002)¹⁵ MANIK and HALOG (2016)²³ CARVALHO et al., (2013)³⁰ GERMANO (2011)¹⁰ GUNKEL et al., (2007)¹¹ BUSTILLO-LECOMPTE et al., (2015)¹⁶ NORFADILAH et al., (2016)²⁴ HOSSAIN et al., (2016)²⁵ AYU and ARYATI (2010)⁵ BLAZY et al. (2015)¹⁷

Depletion of dissolved oxygen in water	+(⁶)	+(¹¹)	+(¹⁹)	+(²¹)	+(³²)	INTANOO et al., (2016) ⁶ THEBALDI et al., (2011) ¹⁹ SHETE and SHINKAR (2013) ³³
Disseminate endemic diseases	-	+(⁹)	-	-	-	CARMO et al. (2013) ¹² WARE and POWER (2017) ¹⁸ KRISHNAN et al. (2016) ²⁶ KOTHARI et al. (2017) ³¹
Release of greenhouse gases	CH ₄ (⁵)	CO ₂ , N ₂ O, CH ₄ +(¹²)	CH ₄ , SO ₂ , NH ₃ +(¹⁸)	+(²⁶)	+(^{31, 32}) CH ₄ , SO _x , NO _x	

*(+) indicates that the effluent generates this impact and the number is the reference *(-) indicates that the effluent does not generate the impact or no related research was found.

2.6 Value-added products of industrial interest that could be produced with effluents

Even today, conventional treatments predominate although they are highly costly (MARTINEZ-BURGOS et al., 2019). However, the macro and micronutrients contained in the effluents and responsible for high organic load can be used to produce different high value-added. With the remarkable advantage about being removed, it is possible to obtain products of wide use, such as metabolites of industrial interest (alcohol, organic acid, solvents, pigments etc.), bioenergy in the form of different types of biogases, biomass of different microorganisms, among others.

However, before defining the type of use of the effluent or metabolite to be produced, the effluent's characteristics must be considered. Thus, effluents with easily fermentable substrates and without toxic compounds such as whey, SV, EMP, POME can be used in the production of metabolites with industrial interest (organic acids, enzymes, proteins, vitamins, etc.), since the fact that they contain compounds that make it difficult the purification process, mainly if the metabolites are intended for food. In the case of CPW, it is rich in easily fermentable carbon sources, however this effluent contains extremely toxic compounds such as hydrocyanic acid (De CARVALHO et al., 2018).

On the other hand, due to the nutrients contained in the effluents, their use as a biofertilizer is also promising. In fact, one of the first applications of effluents was fertirrigation., i.e. the use of effluents as fertilizers in different types of crops to supply soil deficiencies of nitrogen, phosphorus, potassium, etc. Thus, CPW, SV, whey and POME have been used as fertilizers in banana, corn, sugar cane and *Schinus molle* crops, observing positive aspects in said crops (JUNIOR et al., 2012; MAGALHÃES et al., 2014; GÓMEZ and RODRÍGUEZ, 2000; CARRILHO et al., 2016; ABOU-DAHAB et al., 2019). However, these practices have been strongly questioned by the effects that effluents can have on the soil as well as groundwater (ABEGUNRIN et al., 2016; MAGALHAES et al., 2015; CHRISTOFOLETTI et al., 2013b). For example, whey may contain high concentrations of salts and lactic acid, which also affect soil properties (CARVALHO et al., 2013). According to ONYIA et al. (2001) the use of POME as fertilizer could also cause a nutrient imbalance on the soil. Finally, the SH usually contains high pathogenic microbial loads of the order 2.4×10^{10} MPN mL⁻¹ of total coliforms and 7.70×10^9 MPN mL⁻¹ thermally tolerant coliforms (MASSÉ and

MASSE, 2000; BUSTILLO-LECOMPTE and MEHRVAR, 2015; DAVARNEJAD and NASIRI, 2017). Thus, GERBER et al. (2017) found that pig slaughterhouse effluent negatively affects cucumber and lettuce seed germination. Other biotechnological applications of effluents are presented in FIGURE 4. It is observed that the common applications among the effluents are mainly the production energy (hydrogen and methane) and their intermediate metabolites such as organic acids. In fact, other researchers have already signaled that POME contains large amounts of energy and could be harnessed. According AHMED et al. (2015), the amount of energy contained in Malaysia-generated POME would be valued at approximately \$ 1 million.

FIGURE 4 - Different bio-products produced from agro-industrial effluents.



It could be stated that the production of bioenergy (biogas) is one of the most promising alternatives for the effluent utilization since the difference of the other applications has no restriction, besides being an easier process and that does not need complex processes for its purification. Due to the composition complexity of the effluents, some of them contain compounds of toxic nature and others have high microbial loads including pathogenic, which would make it difficult to separate and purify

operations, especially those metabolites intended for food. Also, energy is an input that is needed in the same beneficiation processes and, in addition, anaerobic digestion significantly removes the organic load of said effluents.

According AHMED et al. (2015), MA et al. (2019) the digestion of organic matter is done in four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrogen and methane are specifically produced in acetogenesis and methanogenesis respectively (Moraes et al., 2015). Anaerobic digestion is an interesting way of reusing the effluents, resulting in the production of organic acids, such as propionic acid (HADDADIN et al., 1996), lactic acid (ROUKAS and KOTZEKIDOU, 1991), acetate, and energy in the form of methane and hydrogen (KOTHARI et al., 2017). However, the efficiency of biogas digestion and productivity depends on a large extent in the structure of the microbial community, as well as on environmental factors, such as pH and T (° C), which can be controlled depending on the type of reactor employed.

Different types of bioreactors have been designed for anaerobic digestion and biogas production, from effluents such as up-flow anaerobic filtration, pond system, anaerobic filtration, anaerobic digester, fluidized bed reactor, upflow anaerobic sludge blanket reactor (POH and CHONG, 2009), up-flow anaerobic sludge fixed-film (NAJAFPOUR et al., 2006), expanded granular sludge bed, ultrasonicated membrane anaerobic system, ultrasonic-assisted membrane anaerobic system, membrane anaerobic system, modified anaerobic baffled bioreactor, among others (MORAES et al., 2015; OHIMAIN and IZAH, 2017). The main characteristics of these bioreactors are presented in TABLE 3.

TABLE 3 - Bioreactors used for wastewater treatment and biogas production (H₂ and CH₄).

Type of Bioreactor	Bioreactor features	Retention time	COD removal efficiency	Advantages	Drawbacks	References
Pond system	Depending on the effluents the first ponds are cooling, followed by mixing, anaerobic and facultative. The system can consist of up to 12 ponds.	20-200 days	97%	Low cost; CH ₄ concentration up to 55%; simple and easy operation	Biogas production depends on weather stations; large areas needed; difficult gas capture	Poh and Chong, 2009; Yacob et al., 2006
Anaerobic filtration	Bioreactor with different compartments (airtight vessel, digester and temperature gauge)	15 days	94%	CH ₄ concentration up to 63%; pH and T control (°C)	High cost	Borja and Banks, 1994
Modified anaerobic baffled bioreactor	Device with different compartments (feed tank, magnetic stirrer, peristaltic pump, water jacket)	3-10 days	87-95%	CH ₄ concentration 67-71%; control of variables such as: T (°C) and pH.	High cost	Faisal and Unno, 2001
Fluidized bed reactor	Compact bed system, can be operated with up or down flow	70 days	98-80 %	Large contact area; high organic loading rates; high flow velocity	Needs large amounts of input power.	Borja et al., 2001
Up-flow anaerobic sludge blanket (UASB)	System consisting mainly of pump, sludge blanket, gas separator, gas collector,	Up to 200 days	60-97%	Equipment versatility, can be used for high suspended solids wastewater	Needs long start-up period (2-4 months).	Najafpour et al., 2008; Ohimain and izah, 2017; Chaisri et al., 2007
Anaerobic baffled reactor	Set of series vertical baffles that force effluent to flow over them from inlet to outlet	3-5 days	82-92%	Simple and inexpensive equipment; no moving part or mixing device; CH ₄ concentration 50-75%	Inconvenient, with high viscosity effluents	Faisal and Unno, 2001; Wang et al., 2004; Barber and Stuckey, 1999

TABLE 4 - Bioreactors used for wastewater treatment and biogas production (H₂ and CH₄).

Type of Bioreactor	Bioreactor features	Retention time	COD removal efficiency	Advantages	Drawbacks	References
Up-flow anaerobic sludge fixed-film	Hybrido bioreactor (UASB and upflow fixed film); it is usually composed of pumps, feed tanks, gas separators, brackets, etc.	4-40 days	89-97%	Rapid biotransformation of organic matter; CH ₄ concentration 62-82%	Complex system that needs large amounts of input power.	Najafpour et al., 2008
Continuous stirred tank reactor (CSTR)	It is a mechanically agitated closed tank reactor which increases the contact area with biomass and increases biogas production.	4 days	60-83%	Easy operation; CH ₄ concentration 65-67%	Contains mechanical systems; needs significant amounts of energy	(Irvan et al., 2012;) Ohimain and Izah, 2017; Poh and Chong, 2009)

One of the most widely used systems for effluent treatment and biogas production so far are pond systems; approximately 85% of POME produced in Malaysia is treated with this technology (POH and CHONG, 2009). Another highly employed system is anaerobic baffled reactor, commonly used for the treatment of domestic sewage. In fact, methane plants from effluents already exist today. That is, biogas produced from effluents could play an important energy role in the world's energy matrix. It is noteworthy that each of these technologies can be employed in the production of methane and hydrogen, the main difference is in the inoculum type. Thus, BARROS et al. (2016) produced methane from VW in two up-flow anaerobic bioreactors, using as inoculum sludge from swine wastewater, and obtained 0.18 L CH₄ g⁻¹ COD. In addition, the organic load was reduced by 82%. LALOV et al. (2001), obtained a higher yield (0.33 m³ CH₄ kg⁻¹ COD and a reduction in the organic load of 92%) using a microbial consortium of methanogenic strains.

According to FEROLDI et al. (2014), the effluent from animal slaughterhouses has a high capacity to generate biogas from the organic load and the autochthonous microbial flora. VIDAL et al. (2016), For example, removed 90% of COD, and 60 mL of methane was generated from 320 mL of effluent from cattle and pig slaughterhouses. Besides, WARE and POWER (2017) obtained a yield of 594.59 mL CH₄ g VS⁻¹ (Volatile solids) from poultry slaughter effluent. POME is a highly energetic effluent: according to AHMED et al. (2015), 1 m³ of POME can generate about 28 m³ of biogas, which includes hydrogen.

In Brazil, the production of biogas from the mentioned effluents is a promising alternative, since large volumes of these are generated each year (TABLE 4). Also, they contain a high amount of energy in the form of organic matter (FIGURE 1). Thus, it has been estimated from **Equation 1** that the amount of energy contained in the effluent is about 71,568 GW/h (TABLE 4). That is, approximately 15% of the energy consumed in 2016 (460,829) (MINISTERIO-DE-MINAS-E-ENERGIA DO-BRASIL, 2017), energy that could satisfy the needs of approximately 25 million homes with average consumption of 180 kWh. The TABLE 4 shows that 92% of the energy is contained in VW, because this effluent is generated in greater quantity, besides having extremely high organic loads.

$$E = OL * Cp * V * YE \quad (1)$$

Where: OL: Organic load (g L⁻¹); Cp: Calorific power of methane (CH₄) (802 kJ mol⁻¹); V: Effluent volume (L); YE: Methane yield from effluent (CPW: 0.35 L CH₄ g⁻¹ COD)(UBALUA, 2007); (Whey: 0.23 L CH₄ g⁻¹ COD) (PRAZERES et al., 2012);

(POME: 0.5 L CH₄ g⁻¹ COD)(O-THONG et al., 2012); (Dairy effluent: 0,19 L CH₄ g⁻¹ COD)(Kothari et al., 2017); Slaughterhouse effluent: 0.14 L CH₄ g⁻¹ COD.

TABLE 5 - Amount of energy contained in effluents produced in Brazil.

Effluents	Energy (GWh)	%
CPW	1330.68	1.85
VW	65902.12	92.08
POME	361.44	0.51
SH	1847.62	2.58
Whey	1011.05	1.41

2.7 Conclusion and perspectives

The agro-industrial wastewaters addressed in this manuscript are produced in very large volumes and present high concentrations of organic matter, nitrogen, phosphorus and potassium that can cause great impacts in different ecological systems. The most rational (and economical) way for their disposal is by associating them with the production of high commercial value bioproducts by applying biorefinery concepts. The traditional physicochemical and biological treatment processes can be considered surpassed in a society whose social-economic-environmental development merges with the need to valorize these bioproducts. The production of bioenergy from these industrial bioproducts is the most widely explored pathway for their valorization, especially due to the versatility of the final product and facility of integration into diverse industrial systems.

3. CHAPTER II (The Effect of Hydrolysis and Sterilization in Biohydrogen Production from Cassava Processing Wastewater Medium Using Anaerobic Bacterial Consortia)

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3.1 Abstract

We evaluated the production of bioH₂ from Cassava Processing Wastewater (CPW) using three microbial consortia (*Vac*, *Esg*, and *Lod*) from different Brazilian environments. These consortia consisted of bacteria of the genera *Clostridium*, *Sporanaerobacter*, *Coprococcus*, *Enterococcus*, and others. The CPW was supplemented with nitrogen and used raw or hydrolyzed, and sterilized or not. Four independent variables were optimized (Box-Behnken design): pH, temperature, C/N ratio, and inoculum ratio. Three quadratic models were obtained and explain production of bioH₂ (R² of 0.93, 0.87 and 0.82 for the consortia *Vac*, *Esg* and *Lod*, respectively). The quadratic effects were the most significant in comparison to linear effects and interactions. The optimal conditions were: pH: 5.5-7.0; temperature 37-39°C; inoculum ratio 15%, and C/N ratio 5-3,5. After 48 h, the maximum yields of hydrogen obtained with hydrolyzed and sterilized CPW were 1.82, 1.7 and 1.68 mols of H₂ /mol of maltose for *Lod*, *Esg* and *Vac*, respectively. While, for the only sterilized substrate the yields are in the range 1.33-1.54 mol H₂/mol maltose

Keywords: Cassava processing wastewater, Consortia, Optimization, bioH₂, Dark fermentation

3.2 Introduction

Hydrogen has been envisioned as one of the promising energy sources due to its low impact on the environment and its high energy content (ZIARA et al., 2019). For the utilization of this energetic vector, different types of processes are employed, since this is always associated with a range of compounds (H₂O, carbohydrates, proteins, hydrocarbons, etc.).

According to ZIARA et al. (2019) the main processes for H₂ production are conventional, using fossil fuels as a raw material; and those based on the dissociation of water, thermochemical, electrolysis, photolysis; and biological, using bacteria and microalgae as catalysts for the transformation of biomass. Currently, there is great interest in biological processes that use bacteria (dark fermentation) since the raw materials can be domestic and industrial, solid and/or liquid waste. This bioprocessing result in intense environmental benefits related to the need for disposal and treatment of these wastes.

In the dark fermentation, the use of bacterial mixes presents advantages in relation to the use of pure strains, since they develop mutualistic processes that imply in direct improvements in the production of bioH₂ (GOUD et al., 2012) by better adaptation and linking of biological processes. In fact, most research developed with complex substrates such as CPW will use microbial consortia as inoculum. Like this, (SREETHAWONG and REETHAWONG, 2010) produced bioH₂ using CPW and heat treated sludge as inoculum. LEAÑO and BABEL (2012) observed that physical (sonication) and enzymatic treatments in CPW significantly improved bioH₂ production. Other research focuses on bioreactor type, organic load variation (ANDREANI et al., 2015), or in the optimization of variables such as pH, T, inoculum ratio etc (YIN and WANG, 2019). However, this work focused on the use of different types of media (sterile CPW, non-sterile CPW, Hydrolyzed CPW), the use of different microbial consortia, as well as the calculation of mathematical models.

The effluent has been applied in the production of bioH₂ because it has adequate physical and chemical characteristics (CAPPELLETI et al., 2011) and is produced in large volumes (300-600 L of CPW/ton). Each year approximately 170 billion liters are generated (DE CARVALHO et al., 2018; SILVA et al., 2014).

In addition, the CPW has an enormous polluting potential which is associated with the presence of cyanogen compounds, which if discarded irregularly can cause permanent damage to the local biota (in addition to the risk to human health), and the presence of a high content of organic matter, which allows the initiation of eutrophication processes in the bodies of water (DE CARVALHO et al., 2018). At the same time, the treatment of CPW is costly and the possibility of associating treatment with co-production of energy is of great industrial interest. According to UBALUA (2007) the treatment of CPW is so expensive that it can represent between 20-50% of the total cost of a large scale company. In view of the discussed scenario, there is still paucity of studies on the use of this effluent in the production of bioH₂. Therefore, this work evaluated the generation of bioH₂ using microbial consortiums and CPW treated in different ways as a substrate, adding value to this chain.

3.3 Materials and methods

3.3.1 Consortia sampling

Three samples from different Brazilian environments (TABLE 5) were collected and thermally treated (95 ± 2 °C/ 15 min) aiming to remove methanogens and sulfate-reducing bacteria (WONG et al., 2014), and in the sequence the consortia were cultivated under anaerobiosis. The anaerobic environment was created following the technique described by (BALCH et al., 1979). The removal of the oxygen from the medium was by boiling (100-105 °C) the medium under anoxic conditions (argon atmosphere). The redox potential of the medium was reduced adding NaHCO₃ (1.0 g/L) and Cysteine-HCl (0.5 g/L) at 85 °C and 65 °C, respectively.

All cultures were performed in 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. The maintenance and conservation of the consortia were carried out by weekly transfer to fresh culture media. The inoculum culture medium was composed of: CPW, yeast extract (2.7 g/L), K₂ HPO₄ (2.4 g/L) and KH₂ PO₄ (1.8 g/L).

TABLE 6 - Origins of bacterial consortia in Brazilian environments.

Name	Origin	Location
<i>Lod</i>	CPW sludge	(DMM) -22.301026, -53.361615
<i>Esg</i>	Sewage sludge	(DMM)-25.504002,-49.203573
<i>Vac</i>	Cattle manure	(DMM)-25.549741, -49,224515

3.3.2 Molecular identification of consortia

For each sample, 1000 μ L of consortia culture were centrifuged at 12,000 x g for 1 min. The cell pellet was resuspended in 500 μ L Tris-EDTA, homogenized with 10 μ L of lysozyme solution (20 mg/mL) (Sigma Aldrich, Arklow, Ireland) and incubated at 30°C for 60 minutes. Then, 50 μ L of SDS 10% (w/v) and 10 μ L of proteinase K solution 20 mg/mL (Sigma Aldrich, Arklow, Ireland) were added to the lysis solution, followed by homogenization and incubation at 60 °C for 60 min. To this mixture, 150 μ L of phenol-chloroform (25:24) was added, homogenized by inversion, and centrifuged at 12,000 x g for 5 min. The supernatant was removed, and the DNA was precipitated with 3x (v/v) absolute ethanol. Pellets were washed with 80% ethanol, dried and resuspended in ultrapure water. Total DNA was quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA).

3.3.2.1 Illumina high-throughput sequencing

The V4 variable region of the 16S rRNA gene (bases 515 to 806) was amplified from the total DNA extracted samples according to the Illumina 16S metagenomics sequencing library protocol. DNA was amplified with specific primers for the V3 region containing complementary adaptors for the Illumina platform (CAPORASO et al., 2012) using KlenTAQ polymerase (Sigma Aldrich, Arklow, Ireland). Bar-coded amplicons were generated by PCR under the following conditions: 95 °C for 3 min, followed by 18 cycles at 95 °C for the 30s, annealing at 50 °C for 30s, extension at 68 °C for 60s, final extension at 68 °C for 10 min and hold at 10 °C. Samples were sequenced in the MiSeq platform using the 500 V2 kits, following standard Illumina protocols.

3.3.2.2 Bioinformatics and data analysis

Data generated by the sequencing went through a rigorous quality system that

involved: (I) identification and removal of sequences containing more than one ambiguous base (N); (II) evaluation of the presence and complementarity of primers and barcodes sequences. Chimeric sequences detection, removal of noises from pre-cluster and taxonomic attribution were also performed using standard parameters of QIIME software package, version 1.9.0. By applying the UCLUST method (EDGAR, 2010), sequences presenting identity above 97% were considered the same operational taxonomic units (OTUs) according to the SILVA database (QUAST et al., 2013).

3.3.3 Experimental design and statistical analyses

The substrate used to produce bioH₂ was CPW, provided by the company Amidos São João Ltda. (Nova Andradina, Mato Grosso do Sul, Brazil). The effluent was maintained at -20 °C until use. Initially, CPW was characterized. The sugars content was determined by High-Performance Liquid Chromatography (HPLC).

Anions and cations were analyzed by ion chromatography (761 Compact IC, Metrohm AG). The columns used were Metrosep A Supp 5 - 250/4.0 and Metrosep C 3250/4.0 for anions and cations respectively. In the case of anions the mobile phase was Na₂CO₃ 3.2 mM and NaHCO₃ 1.0 mM at a flow rate of 0.7 mL/min and for the cations, HNO₃ 3.5 mM at a flow rate of 0.9 mL/min. Run times were 30 and 25 min for anions and cations, respectively. The yield (mol H₂/mol maltose) was calculated using the ideal gas equation.

Finally, the determination of nitrogen, COD (chemical oxygen demand), total volatile solids, total fixed solids were done using Standard Methods for the Examination of Water and Wastewater American-Public-Health-Association 1992.

3.3.3.1 Selection of variables

3.3.3.1.1 Nitrogen source

Each consortium was evaluated using three nitrogen sources (yeast extract, urea, and ammonium sulfate) at C/N:3.3 (CHENLIN and HERBERT, 2007). The experiment was performed at 35°C, pH 7,0 and inoculum rate 20%. The pH of the media was adjusted with 1.0 M NaOH solution and 1.0 M HCl. A one-way ANOVA test ($p \leq 0.1$) was performed for the selection of the nitrogen source with Fisher's posterior test.

3.3.3.1.2 Optimization

Five input variables were evaluated, four quantitative, pH (4-9), T (30 - 42°C), C/N (1-10), % inoculum (0%-20%) (2×10^6 - 5×10^6 CFU/mL), and qualitative (hydrolysis or not of CPW) by Placket-Burman (PB) design. The Pareto Diagram (not shown) was used to determine the significance of the effects. Three PB design with 12 experimental runs, one for each consortium were made. Hydrolysis of CPW was performed using 1% (v/v) HCl at 121 °C/ 1 h. Variable selection and levels were based on previous work. For example, YIN and WANG, (2019) demonstrated that the variables pH, T and % Inoculum significantly affect the production of bioH₂. Besides, LI and FANG, (2007) will indicate that the C/N ratio also affects the production of bioH₂. Finally, O-THONG et al. (2011) indicated that the acidic or enzymatic hydrolyses can convert the starch contained in the CPW into glucose or maltose, which would directly affect the dark fermentation. Once the significant parameters were defined, the variables selected for each consortium were optimized using a Box-Behnken (BB) planning with four factors and three central points. TABLE 6 shows the matrix of the 27 experimental cases for each consortium, the data of the three optimizations were adjusted to a quadratic model presented in equation 1.

$$H_2 = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{K-1} \sum_{j=i+1}^K \beta_{ij} X_i X_j \quad \text{EQUATION 2}$$

Where β_0 , β_i , β_{ii} and β_{ij} are the constant, linear, squared and interactive coefficients of the input parameters, respectively. Where (i = 1-4), are the four independent variables (X_1 : temperature; X_2 : pH; X_3 : inoculum; X_4 : C/N), and H₂ is the accumulated production of bioH₂. All statistical analyses 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.

The CPW, besides being supplemented with the nitrogen source, was also supplemented with 2.4 g/L K₂HPO₄ and 1.8 g/L KH₂PO₄ (CAPPELLETTI et al., 2011). Initially, the optimization of the bioH₂ production was done using the M-medium (sterile CPW), after which the optimized variables were used to evaluate the bioH₂ production using M medium, H medium (hydrolyzed and sterile CPW) and B medium (non-sterile CPW). A control (Co) was made which was neither sterilized nor inoculated.

TABLE 7 - BB design matrix for the consortia *Vac*, *Esg* and *Lod*, experimental and predicted results for bioH₂ production.

Run	T °C	pH	% Ino.	C/N	<i>Vac</i> (mL bioH ₂)		<i>Esg</i> (mL bioH ₂)		<i>Lod</i> (mL bioH ₂)	
					H ₂ exp	H ₂ pred	H ₂ exp	H ₂ pred	H ₂ exp	H ₂ pred
1	0	1	0	-1	0.44	0.43	0.43	0.37	0.450	0.403
2	0	1	1	0	0.55	0.53	0.48	0.43	0.470	0.440
3	0	-1	1	0	0.34	0.38	0.25	0.25	0.350	0.330
4	1	0	-1	0	0.19	0.10	0.15	0.10	0.100	0.050
5	-1	0	-1	0	0.27	0.14	0.29	0.21	0.150	0.150
6	0	-1	0	-1	0.32	0.28	0.25	0.20	0.324	0.310
7	1	0	0	-1	0.23	0.29	0.19	0.22	0.350	0.320
8	-1	1	0	0	0.4	0.41	0.41	0.45	0.370	0.380
9	0	0	1	1	0.64	0.61	0.44	0.35	0.410	0.320
10	-1	0	0	-1	0.35	0.44	0.38	0.40	0.550	0.470
11	0	0	0	0	0.83	0.86	0.45	0.49	0.550	0.740
12	0	1	-1	0	0.08	0.10	0.18	0.20	0.050	0.076
13	-1	-1	0	0	0.28	0.27	0.25	0.24	0.350	0.320
14	0	0	1	-1	0.57	0.43	0.41	0.40	0.400	0.480
15	0	0	-1	1	0.11	0.23	0.19	0.17	0.180	0.080
16	0	0	0	0	0.87	0.86	0.49	0.49	1.039	0.740
17	0	-1	-1	0	0.05	0.11	0.02	0.07	0.030	0.062
18	0	1	0	1	0.5	0.50	0.25	0.20	0.280	0.297
19	0	-1	0	1	0.53	0.49	0.13	0.19	0.200	0.250
20	0	0	0	0	0.88	0.86	0.52	0.49	0.620	0.740
21	1	-1	0	0	0.41	0.38	0.18	0.11	0.331	0.300
22	1	0	1	0	0.45	0.53	0.14	0.23	0.450	0.450
23	-1	0	1	0	0.35	0.39	0.43	0.49	0.350	0.400
24	1	0	0	1	0.67	0.63	0.18	0.17	0.280	0.365
25	1	1	0	0	0.4	0.40	0.22	0.20	0.350	0.360
26	0	0	-1	-1	0.11	0.12	0.12	0.18	0.010	0.085
27	-1	0	0	1	0.4	0.38	0.41	0.38	0.230	0.260

* mL bioH₂ produced / 5 ml medium. The lower and upper data are presented with -1 and 1 respectively, and the midpoint with "0". *Vac*: pH (4-8), T °C (34-44), % Inoculum. (10%-20%), C/N (2-8); *Esg*: pH (4.5-6.5), T°C (32-42), %Inoculum. (10-20), C/N (3-5); *Lod* : pH (4.5-6.5); T °C (32-42), %Inoculum (10-20), C/N (2.0–5.0).

3.3.4 Microbial kinetic profile

Samples of 200 µL of the fermented media were collected periodically (8h, 16h, 24h, 36h, and 48h), diluted and plated on MRS (De Man, Rogosa and Sharpe medium), using the Pour Plate method. The samples were anaerobically incubated at the optimum temperatures determined for each consortium in GasPak jars with ANAEROBAC kit during 24 h.

3.3.5 Kinetic profile of bioH₂ production

Biogas production was periodically analyzed (8h, 16h, 24h, 36h, and 48h). The gas produced was collected in glass syringes (5 mL and 100 mL) and analyzed on a gas chromatograph 490 Micro GC System (Agilent), equipped with two columns (Molsieve 5Å and PoraPLOT U) and a thermal conductivity detector (μ TCD). In the Molsieve 5Å, the injection temperature was 110°C, the injection time 20 ms, column temperature 90 °C, and initial pressure 190 kPa. In column PoraPLOT U (PPU), the injection temperature was 110°C, column temperature 90°C and initial pressure 150 kPa. The peak retention time for H₂ was 0.4 min (Ms5A) and for CO₂ and CH₄ were 0.405 and 0.425 respectively in the PPU. The time of each run was 1.2 min. The mobile phase used was argon with a purity of 99.999%.

3.3.6 Analyses of organic compounds

The organic compounds of the fermentative process were analyzed using HPLC. Before injecting, samples (1.5 mL) were centrifuged at 6000 rpm (10 min) and subsequently microfiltered on cellulose acetate membranes (0.22 μ m). It was used a chromatograph Agilent 1260 Infinity Quaternary LC with RI detector and Hi-Plex-H column. Detector and column temperatures were 50 °C and 60 °C, respectively. The mobile phase was 5mM H₂SO₄ at a flow rate of 0.6mL/min and the injection volume were 10 μ L. Standard curves were constructed to quantify sugars, acids, and alcohols (concentrations 0.01g/L-1.5g/L).

3.4 Results and Discussion

The physicochemical characterization of CPW is shown in TABLE 7. It has been found different nutrients in the effluent, such as maltose, macro, and micronutrients. This makes it a suitable residue for dark fermentation, for the production of bioH₂. In fact, according to LUCAS et al. (2015) the CPW is an ideal substrate for the bioprocesses due to their characteristics, which could require little or no type of supplementation. In this sense, DE CARVALHO et al. (2018) have shown that CPW is a source of carbohydrates, proteins, phosphates, and potassium, which makes it suitable to use for the production of biogas, ethanol, other fermented products and also to produce microalgal biomass.

AMORIM et al. (2018) also considered that the CPW is a suitable substrate for the development of biotechnological processes, such as the production of bioH₂ and carboxylic acids due to the high content of organic matter of easy degradation.

TABLE 8 - Physical-chemical composition of CPW.

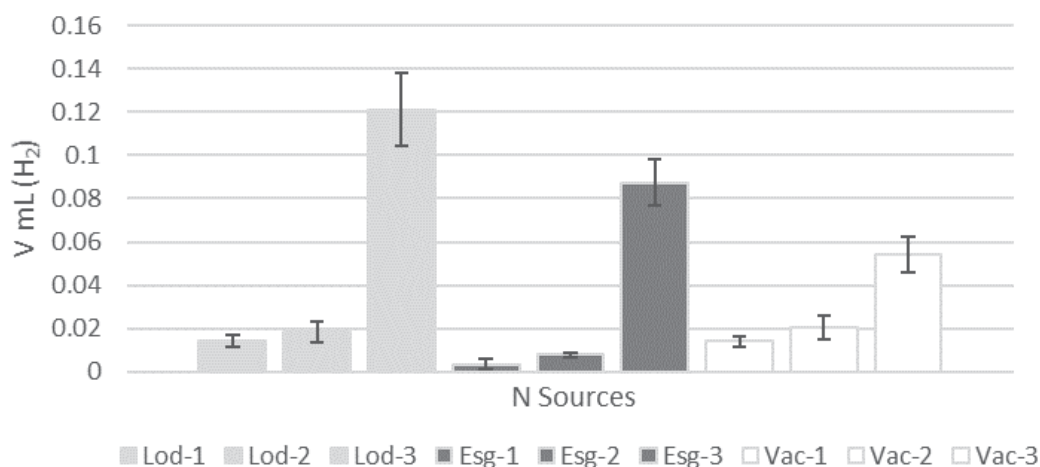
Parameter	mg/L	Parameter	mg/L
Cl	52	Na	16.64
Br	30	NH ₄	61.53
NO ₃	4.0	K	435.75
PO ₄	47.30	Mg	43.08
SO ₄	63.77	Ca	86.49
COD (mg O ₂ /L)	8488 ± 363	Total nitrogen (Kjeldahl)	180
pH	5.5	Volatile solids	3500±500
Maltose	1300	Fixed solids	1900±400
Glucose*	600		
Maltose*	1500	Total solids	5400±400

*Only hydrolyzed CPW

3.4.1 Selection of the nitrogen source

The results showed that nitrogen source type has a significant effect ($p \leq 0.1$) in the bioH₂ production for the three evaluated consortia (*Lod*, *Esg* and *Vac*). The p-value for each consortium was 0.018; 0.029 and 0.012 respectively. In all consortia, the highest production of bioH₂ was obtained with the yeast extract and the lower production with urea (FIGURE 5). Probably this behavior is because the yeast extract contains essential amino acids and micronutrients that stimulate the bacterial growth and the production of bioH₂.

FIGURE 5 - BioH₂ produced from the different sources of nitrogen.



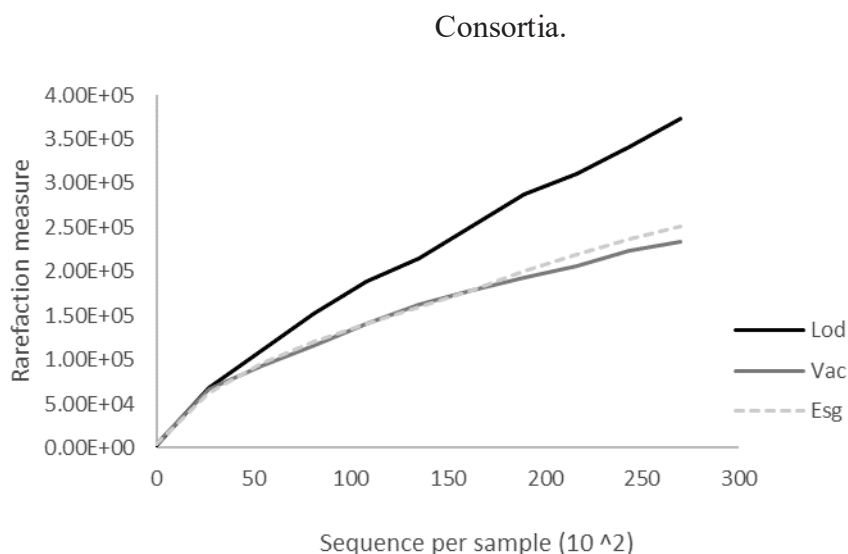
* N Sources: 1 Urea; 2: Ammonium sulphate; 3: Yeast extract

On the other hand, urea releases ammonia, especially during autoclaving, causing a toxic effect on microorganisms (CHEN et al., 2018; SOLLI et al., 2018) and inhibiting the production of bioH₂ (KESKIN et al., 2018). Ammonium sulfate was the second best source, however, with better results than with urea but still much lower than that obtained using yeast extract. CAPPELLETTI et al. (2011) also used (NH₄)₂SO₄ as the nitrogen source, but supplemented the medium with other compounds containing Mg, K, Fe, Ca, Zn, Mn, Cu etc. AMORIM et al. (2014) used CH₄N₂O as the source of N and also supplemented with other compounds of Ni, Fe, Ca, Co, Se, K and P. For their part ZAMPOL et al. (2015) they used yeast extract as a source of N and only supplemented with compounds of K and Mg, Na and vitamin B₁₂. This is because the stated compounds are nitrogen sources, much poorer in other nutrients than the yeast extract. According to LUCAS et al. (2015) the micronutrients and trace elements are indispensable for the maintenance, required by metabolism and for cell growth. The problem of using the yeast extract relies on its higher cost in comparison to the other sources evaluated, compensated by the intense increase in production. Thus, the optimization of bioH₂ production was performed using yeast extract.

3.4.2 Molecular identification of consortia

The rarefaction curves indicated that there was good coverage of the bacterial community present in each consortium (FIGURE 6). Importantly, in *Lod* consortium, the highest number of operational taxonomic units (OTUs) were obtained.

FIGURE 6 - Rarefaction curve for the bacterial community consortia *Lod*, *Vac*, *Esg*.



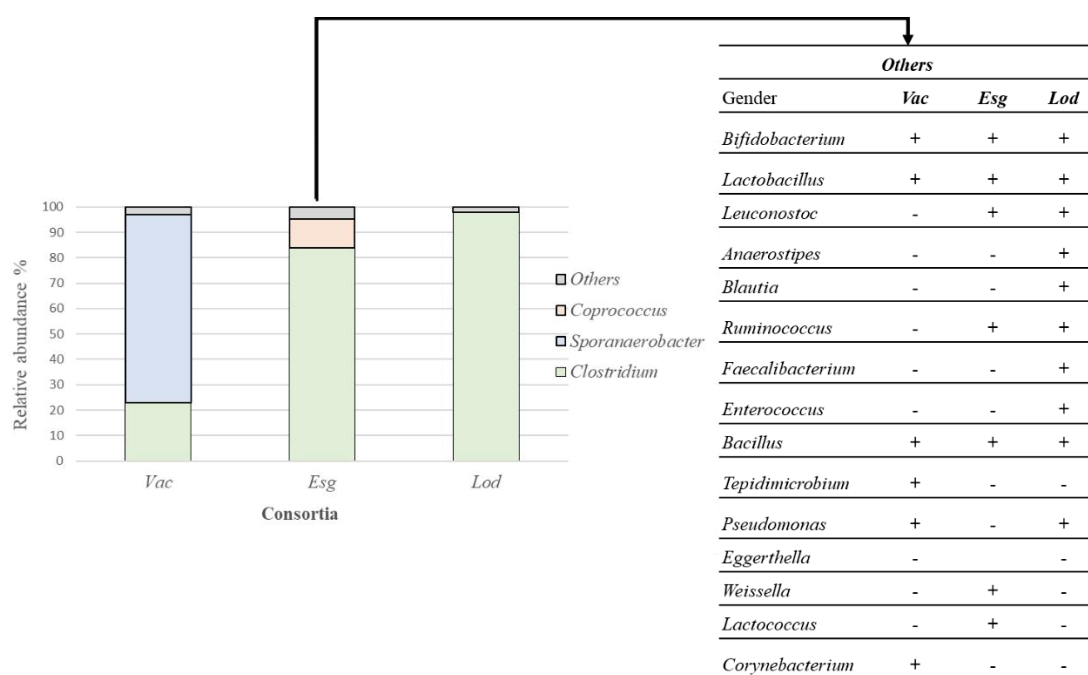
The *Vac* consortium consisted mainly of the genera *Sporanaerobacter* 74% and *Clostridium* 23%, other genres 3% (Figure 7).

The *Sporanaerobacter* are usually strict anaerobes, with demonstrated ability to sporulate (EUGENIO et al., 2002) and produce bioH₂ (LI et al., 2018). *Sporanaerobacter acetigenes*, for example, can use glucose as a source of energy and produce acetate, isobutyrate, isovalerate, bioH₂, and CO₂ (EUGENIO et al., 2002). On the other hand, the genus *Clostridium* also has a significant presence, being one of the most studied genera in the production of bioH₂ (KESKIN et al., 2018; SYDNEY et al., 2018) due to its different metabolic pathways that allow to use varied types of carbohydrates (SIKORA et al., 2013). Being strict anaerobes and spore producers are the main characteristics of this genus (HALLENBECK, 2013), consequently, they are resistant to adverse conditions. Among the species reported in the literature as producers of bioH₂, *Clostridium saccharoperbutylacetonicum*, *C. tyrobutyricum*, *C. butyricum*, *C. acetobutyricum*, *C.*

beijerinckii, *C. thermolacticum*, *C. thermocellum*, and *C. paraputrificum* (BALACHANDAR et al., 2013) are the major ones.

The **Esg** consortium consisted of 84% *Clostridium*, 11% *Coprococcus* and 5.0% others (FIGURE 7). *Coprococcus* are generally found in human feces, being gram-positive, anaerobic coconut-shaped bacteria with the ability to ferment carbohydrates and, in some cases, produce bioH₂ and volatile fatty acids (HOLDEMAN and MOORE, 1974). On the other hand, the **Lod** consortium consisted mainly of *Clostridium* 98% and 2.0% by other genus (FIGURE 7).

FIGURE 7 - Microbial composition for the Vac, Esg and Lod consortia. Genetic analysis based on the V4 region of the 16s rDNA gene.



3.4.3 Selection other variables and optimization of bioH₂ production

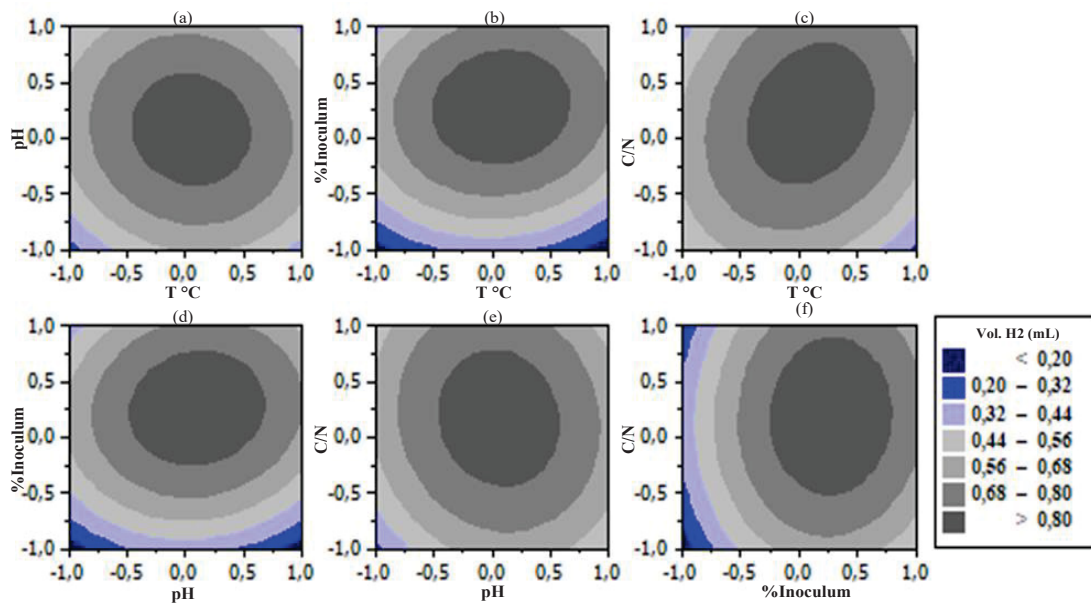
The Pareto Diagram of PB indicated that all variables evaluated had significant influence ($p \leq 0.05$) on bioH₂ production for the three **Lod**, **Esg** and **Vac** consortia. Even though the qualitative variable (hydrolysis or not of the CPW) was significant ($p \leq 0.05$), the optimization was only done with the CPW without hydrolysis.

The optimizations performed with the Box-Behnken (BB) design, TABLE 6 shows experimental and predicted values, which were used for the construction of response surface graphs and mathematical models.

3.4.4 *Vac*, *Esg* and *Lod* optimizations

The response surface graphs for the optimization of input variables and production of bioH₂ for *Vac* consortia are presented in FIGURE 8. The optimization was performed using the following ranges, pH (4-8), T °C (34-44), % Inoculum (10%-20%) and C/N (2-8) (TABLE 7).

FIGURE 8 - Projection of response surfaces for bioH₂ production by *Vac* consortium as a function of pH (4-8), T °C (34-44°C), Inoculum ratio (10%-20%), and C/N ratio (2-8); coded values.



The results showed that the maximum production of bioH₂ is within the selected ranges. In fact, according to the graphs of interactions of the variables (FIGURE 8), it is possible to obtain the maximum bioH₂ production in the T intervals between 36.5-41.5°C, pH between 5-7%, Inoculum between 13.75% -17.5% and C/N 3.5-7.5.

However, the optimal conditions of the variables were selected so that they were centralized (central point) and thus ensure that the process is in the region optima. It is not advisable to select boundary conditions since it increases the chances of minor disturbances in the process resulting in large changes in production. It was determined

that the optimum temperature and pH in the *Vac* were 39 °C and 6.0, respectively, because in this consortium the genus *Sporanaerobacter* predominates (74%), which, according to EUGENIO et al. (2002) and REZGUI et al. (2012) are moderately thermophilic with an optimal growth temperature of 40 °C. As the *Sporanaerobacter*, bacteria of the genus *Clostridium* (23% of the consortium composition) can also develop such conditions.

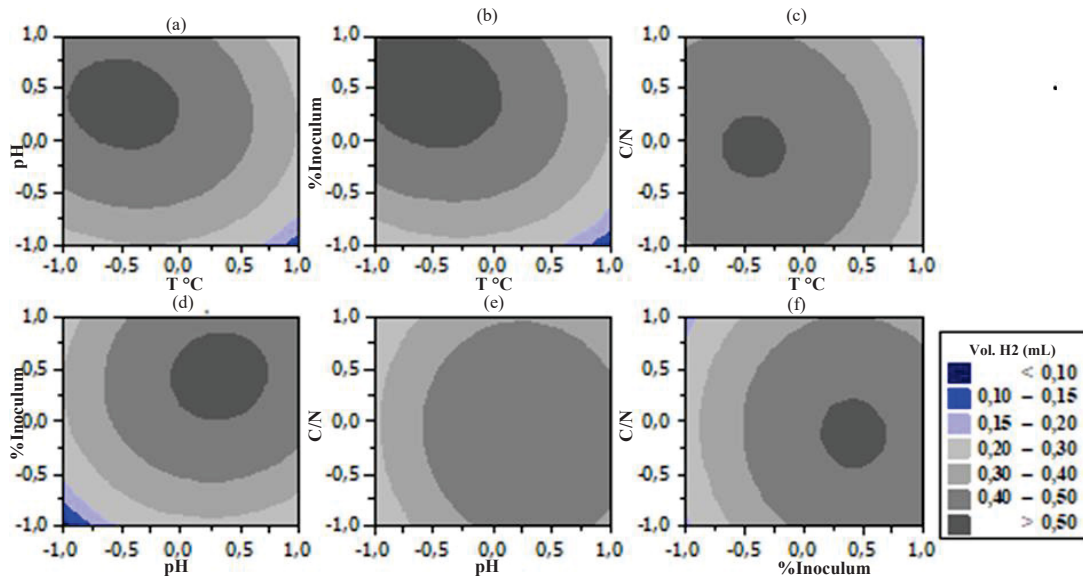
The intervals of the input variables for the *Esg* and *Lod* consortia were, T (32°C-42 °C), pH (4.5-6.5), %Inoculum (10%-20%) and C/N (3,0 -5,0) and T (32 °C -42 °C), pH (4.5-6.5), %Inoculum (10%-20%) and C/N (2.0 - 5.0), respectively (FIGURE 9 and 10).

Regarding the % inoculum variable, a similar behavior was observed for the consortia *Vac* and *Lod*, in which a concentration above 15% showed no increase in bioH₂ production.

However, for the *Esg* consortium, an inoculum rate above 16% was required since below this value the process is outside the optimal region (FIGURES 9b, 9d, and 9f). YIN and WANG (2019) found that above 13% of inoculum did not improve the production of bioH₂ with *Enterococcus faecium* INET2.

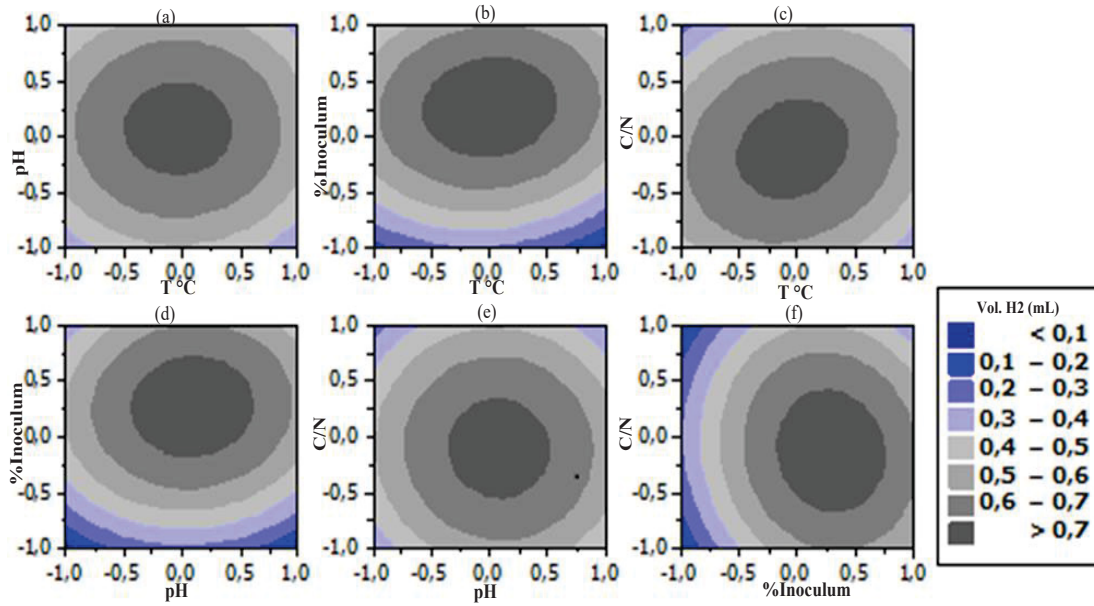
As regards the C/N variable (LI and FANG, 2007) evaluated that a wide range from 3.3 to 130 significantly affect bioH₂ productivity. However, this parameter depends on the organisms involved and the composition of the culture medium, and which should be optimized on a case-by-case basis. In this sense, it can be inferred that the optimum C/N depends on the type of microorganism, as well as on the consortium and the source of N employed. In this research the optimal C/N for the *Vac*, *Esg*, and *Lod* consortia were 5.0; 4.0 and 3.5 respectively, being these C/N close to those reported by other authors (MORIMOTO et al., 2004; LIU and SHEN, 2004).

FIGURE 9 - Projection of response surfaces for bioH₂ production by *Esg* consortium as a function of pH (4.5-6.5), T °C (32-42°C), Inoculum ratio (10%-20%), and C/N ratio (3-5); coded values.



The optimal region for the *Vac* and *Esg* consortiums is at the selected levels. However, the optimal region is better defined in the *Lod* consortium (FIGURE 10). In the *Esg* consortium, the optimum temperature range agrees with the minimum limit (FIGURES 9a and 9b). The ranges of the variables in which the production of bioH₂ by the *Esg* consortium is maximized are: T (33-37 °C), pH (5.3-6.25), % inoculum (15%-20%) and C/N (3.5 - 4.25); while for the consortium *Lod* were: T (34.5-39.5 °C), pH (5.25-6.25), %inoculum (13.75%-18.75%) and C/N (3.125 - 4.25). In the *Esg* and *Lod* consortia, the predominant genus was *Clostridium*, which explains the fact that in both consortia the optimal temperature and pH ranges were nearby. However, the optimal temperature and pH of the *Esg* consortium were 35 °C and 6.0, while for the *Lod* consortium it was 37 °C and 5.5. The pH values found to agree with those reported by (VALENTIN-REYES et al., 2018) which showed, for example, that *Clostridium beijerinckii* can decrease its metabolic activity at pH below 5.5. Thus, the optimum growth temperatures of *Lod* and *Esg* consortia composed mostly of *Clostridium* were in the range of 35-37°C. KAO et al. (2014) in their bioH₂ production work with *Clostridium butyricum* and *Rhodospseudomonas palustris* also used an incubation temperature of 37 °C. CHIN et al. (2003) also established 37° C as the growth temperature of *Clostridium acetobutylicum*.

FIGURE 10 - Projection of response surfaces for bioH₂ production by *Lod* consortium as a function of pH (4.5-6.5), T °C (32-42°C), Inoculum ratio (10%-20%), and C/N ratio (2-5); coded values.



3.4.5 Mathematical Models

The three mathematical models found for the consortiums *Vac*, *Esg* and *Lod* are presented in equations 2, 3 and 4 respectively.

$$H_2 = 0.859 + 0.02533X_1 + 0.0369X_2 + 0.17366X_3 + 0.06926X_4 - 0.23959X_1^2 - 0.25146X_2^2 - 0.32533X_3^2 - 0.18095X_4^2 + 0.0975X_1X_4 \quad \text{Equation: 2 (Vac)}$$

$$H_2 = 0.48693 - 0.09228X_1 + 0.07473X_2 + 0.10139X_3 - 0.01518X_4 - 0.10517X_1^2 - 0.12821X_2^2 - 0.12176X_3^2 - 0.08747X_4^2 \quad \text{Equation: 3 (Esg)}$$

$$H_2 = 0.736333 - 0.01158X_1 + 0.032083X_2 + 0.159167X_3 - 0.042X_4 - 0.177833X_1^2 - 0.216083X_2^2 - 0.2917X_3^2 - 0.2024X_4^2 \quad \text{Equation: 4 (Lod)}$$

Where: X₁: temperature; X₂: pH; X₃: inoculum; X₄: C/N

The results obtained with the three consortia follow a normal distribution. In addition, they fulfill the assumption of homoscedasticity and independence of variable (good dispersion of residues) (results not shown). The main characteristics of the models are presented in TABLE 8, in which it is possible to emphasize that the squares of all the variables were significant ($p \leq 0.05$). In the particular case of the *Vac* model, the interaction X₁X₄ also proved to be significant ($p \leq 0.05$). On the other hand, it is possible to observe that in the models there are terms that were not significant ($p \leq 0.05$). Usually,

because of the interactions among the different variables, there is a lack of adjustment that can be attributed to the large number of variables that were optimized (YIN and WANG, 2019).

TABLE 9 - Characteristics and parameters of mathematical models for the production of bioH₂ by *Lod*, *Esg* and *Vac*.

Source	Model <i>Vac</i> F- value	P- value	Model <i>Esg</i> F- value	P- value	Model <i>Lod</i> F- value	P- value	Model <i>Lod</i> R ²	82.64%
A ₀	16.955	0.000	11.640	0.000	9.871	0.000	Adj R ²	62.38%
X ₁	0.993	0.171	-4.412	0.001	-0.311	0.761	Pred R ²	42.53%
X ₂	1.457	0.171	3.572	0.004	0.860	0.407		
X ₃	6.855	0.000	4.847	0.000	4.267	0.001		
X ₄	2.734	0.018	-0.726	0.482	-1.126	0.282	Model <i>Esg</i>	
X ₁ ²	-6.305	0.000	3.352	0.006	-3.178	0.008	R ²	87.63%
X ₂ ²	-6.618	0.000	-4.086	0.002	-3.862	0.002	Adj R ²	73.20%
X ₃ ²	-8.562	0.000	-3.881	0.002	-5.214	0.000	Pred R ²	30.30%
X ₄ ²	-4.762	0.000	-2.788	0.016	-3.619	0.004		
X ₁ X ₂	-0.752	0.466	-0.852	0.411	-0.004	0.997	Model <i>Vac</i>	
X ₁ X ₃	1.026	0.325	-1.063	0.309	0.580	0.572	R ²	93.15%
X ₁ X ₄	2.222	0.046	-0.290	0.777	0.967	0.352	Adj R ²	85.15%
X ₂ X ₃	0.997	0.338	0.458	0.655	0.387	0.706	Pred R ²	60.86%
X ₂ X ₄	-0.855	0.409	-0.374	0.715	-0.178	0.862	P-value < 0.05	
X ₃ X ₄	0.393	0.702	-0.276	0.787	-0.619	0.547		

*Where: X₁: temperature; X₂: pH; X₃: inoculum; X₄: C/N

In the models, it was found that the interactions T and pH were not significant. Similar behavior was observed by (YIN and WANG, 2019) in the optimization of bioH₂ production using dairy effluents as substrate and activated sludge as inoculum, as well as (YIN and WANG, 2019) in the optimization of bioH₂ production from *Enterococcus faecium* INET2 with glucose as substrate. However, WANG et al. (2013) found that T and pH interactions were significant in the production of bioH₂ using cow dung as an inoculum. In addition, HYE et al. (2008) using *Clostridium tyrobutyricum* JMI also observed significant effect on the interaction T and pH. This fact can infer that this interaction is affected by the type of inoculum (YIN and Wang, 2019).

The R² coefficients for the *Vac*, *Esg* and *Lod* models were 0.93, 0.87 and 0.82 respectively, indicating that the models have a high correlation. The adjusted models (adjust R²) have the ability to predict 85.15%, 73.20% and 62.38% of the accumulated bioH₂ production. It is possible to say that the accumulated production of bioH₂ is mainly governed by the selected variables. Thus, it is plausible to attribute 7%, 13% and 18% of

the variability in the *Vac*, *Esg* and *Lod* models, respectively, to external factors that were not controlled.

In fact, it is observed that the three statistical models present good agreement between the experimental and predicted values (TABLE 9). At the same time, it can be confirmed that the models are suitable for simulating the production of bioH₂ at the established intervals. According to the *Vac* model the optimum conditions are T: 39.6 °C; pH: 6.14; % inoculum: 16.4 and C / N: 5.69. In terms of the *Lod* model, the optimal conditions for the T and C/N variables agree with the central points, while for the % inoculum and pH variables were 16.35% and 5.59, respectively. Finally, according to the *Esg* model, the optimal conditions were T: 35 °C; pH: 5.82; % inoculum: 17.2 and C / N: 4.0. The experimental volumes of bioH₂ and predicted were within the variability (TABLE 9), except the value predicted by the *Vac* model.

TABLE 10 - Comparison between the volumes of bioH₂ produced in the optimum experimental conditions and the models

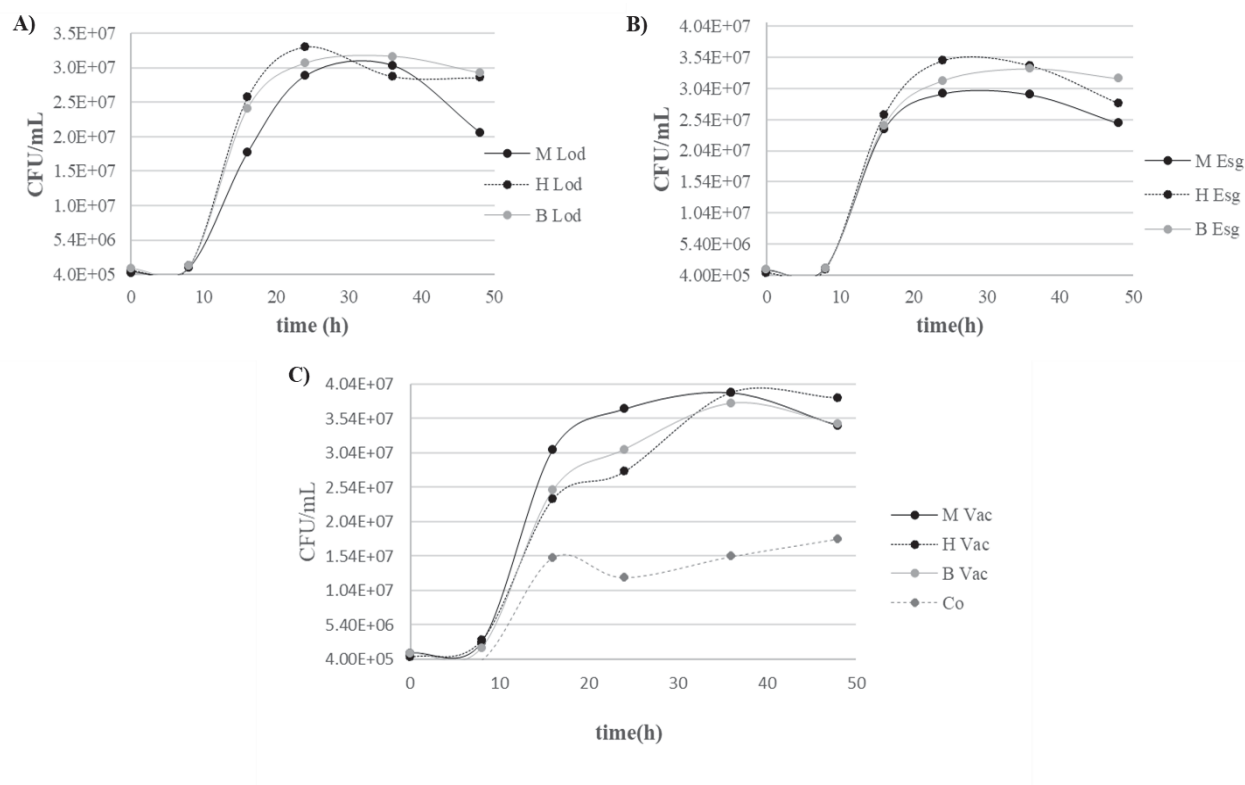
Consortium	BioH ₂ (mL)	
	Experimental	Predicted value
<i>Vac</i>	0.64±0.056	0.89
<i>Lod</i>	0.74±0.10	0.76
<i>Esg</i>	0.55±0.07	0.52

In the case of the *Lod* and *Esg* models, there were no significant differences between the predicted and obtained bioH₂ values. However, in the *Vac* consortium, the predicted bioH₂ volume was 25% higher than the volume obtained experimentally. This result was not expected but could be attributed to factors that were not fully controlled.

3.4.6 Analysis of bioH₂ production under optimum conditions

The kinetic growth profile of the biomass reaches the stationary phase in 24 hours in all cases (FIGURES 11) except Co (Control), which is still in the exponential phase (FIGURE 11C). In addition, it is possible to observe a slightly higher growth of the consortia in the H medium which could be explained by the bioavailability of the substrate. The bioH₂ production performed under the optimized conditions described above ceased within 48 h for all consortia.

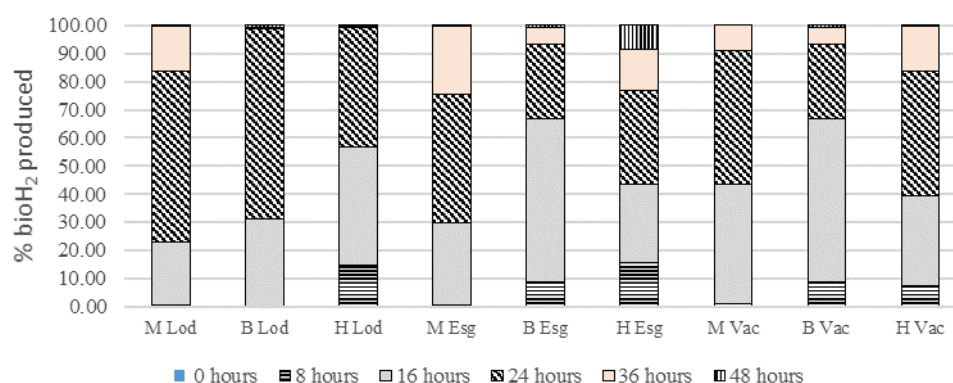
FIGURE 11 - Microbial growth profile (A) *Lod* consortium, (B) *Esg* consortium, (C) *Vac* consortium.



* M: CPW sterilized; B:CPW Non-sterile; H:CPW Hydrolyzed and sterile.

The production of bioH₂ between 36 and 48 hours was very little for all consortia (FIGURE 12), except **H-*Esg*** which produced 8.0% of the total accumulated bioH₂ even though it was in the stationary phase (Figure 11B). In the dark fermentation processes, the bioH₂ is mainly generated in the exponential phase of the microbial growth since its production is directly linked to the maintenance of the redox balance during the production of cellular energy.

FIGURE 12 - Kinetic profile of bioH₂ production with *Vac*, *Esg* and *Lod* consortia.



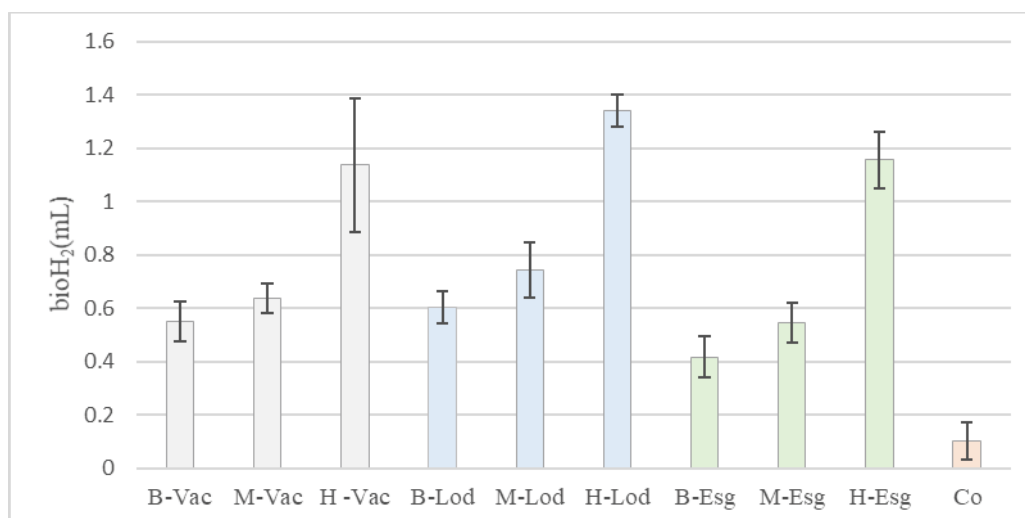
* M: CPW sterilized; B: CPW Non-sterile; H: CPW Hydrolyzed and sterile

The bioH₂ in the dark fermentation processes is generated mainly in the exponential phase of the microbial growth since its production is directly linked to the maintenance of the redox balance during the production of cellular energy. According to BALACHANDAR et al. (2013); HAN and SHIN, (2004) in the stationary phase, there is low or no bioH₂ production.

In the kinetics of bioH₂ production, it is also possible to observe that in 8 hours only hydrolyzed-media presented a significant amount of accumulated bioH₂. Thus, for example, **H-Lod**, **H-Esg**, and **H-Vac** generated 14.6%, 15.5 % and 7.5% of the total bioH₂, respectively, in this interval of time. This can be explained by the presence of glucose traces as the product of acid hydrolysis, which can be fermented more easily than maltose (CHIN et al., 2003; DAS and VEZIROGLU, 2008; GUO et al., 2010; SAADY, 2013). The production of bioH₂ reached around 80% of bioH₂ in 24 h and 98% of bioH₂ in 36 h for all consortia.

The total volumes of bioH₂ produced are shown in FIGURE 13, where it is possible to conclude that in the **H** medium the production is higher, showing that the hydrolysis favored the bioavailability of the sugar fractions that were initially present in the form of starch.

FIGURE 13 - Cumulative volume of bioH₂ produced by different consortia and culture media.



* M: CPW sterilized; B: CPW Non-sterile; H: CPW Hydrolyzed and sterile

Media M and B had no free glucose, and 1.3 g/L of maltose; while after hydrolysis the H-medium maltose increased to 1.5g/L and glucose to 0.6 g/L, resulting in 1.8 g/L maltose-equivalent. However, hydrolysis requires more energy, and the use of chemical reagents, such as HCl or H₂SO₄.

The yield of bioH₂ from the substrate was variable ($Y_{H_2/s}$), depending on the consortium. However, the highest $Y_{H_2/s}$ were obtained in H medium (1.82, 1.7 and 1.68 mol H₂/mol of maltose by *Lod*, *Esg* and *Vac* consortia, respectively).

The unhydrolyzed media (sterilized and unsterilized) showed no significant differences ($p \leq 0.05$) between them. However, it was possible to observe a trend of higher production of bioH₂ in cultures with M medium (FIGURE 13). The behavior can be explained by the elimination of the natural microbiota from the effluent and consequent decrease of competition for the substrate. In the control (Co), which was neither sterile nor inoculated, 0.10 ± 0.07 mL of bioH₂ was obtained. Thus, it can be inferred that non-sterile CPW also contains small amounts of bioH₂ producing bacteria.

In media M, the yield of bioH₂ for the *Vac* and *Lod* consortia ranged from 1.33-1.54 mol H₂/mol maltose. The lowest yield was for the *Esg* consortium (1.14 mol H₂/mol maltose). On the other hand, for media B the yields were 1.25, 1.14 and 0.87 mol H₂/mol maltose for the *Lod*, *Vac* and *Esg* consortia, respectively.

In the process of dark fermentation, multiple reactions occur in the different metabolic routes, especially when microbial consortia are used. This set of reactions depends on the

On the other hand, the productivity (mL H₂ / L medium / h) in the H medium was 5.62, 4.83 and 4.75 for *Lod*, *Esg* and *Vac* respectively. In addition, in media B it was, 2.8, 2.5 and 2.0 for *Lod*, *Vac*, and *Esg* respectively. LUCAS et al. (2015) in their work with CPW achieved a bioH₂ productivity of 1.51 mL/H₂/L medium/h, approximately 50% lower than that obtained in media B. REUNGSANG et al. (2006) reached half a productivity (1.4 mLH₂/L medium/h) using the same substrate, an even lower yield than that obtained by (LUCAS et al., 2015). ZAMPOL et al. (2015) obtained a maximum yield of 36 mmol bioH₂/L, however, the fermentation time was 180 h. In this work, a maximum yield of 10.61 mmol bioH₂/L was obtained with the best consortium (*Lod*) in H-medium in 48 hours, in other words, in 180 hours approximately 4 batches could be obtained (approximately 42 mmol of bioH₂). According to AMORIM et al. (2014) the production of biologically hydrogen (bacteria) is attractive because it takes advantage of waste and has a lower impact on the environment than methods that use fossil fuels as substrates or those based on water molecule dissociation such as electrolysis. Furthermore, biological processes need less input energy (MANISH and BANERJEE, 2008).

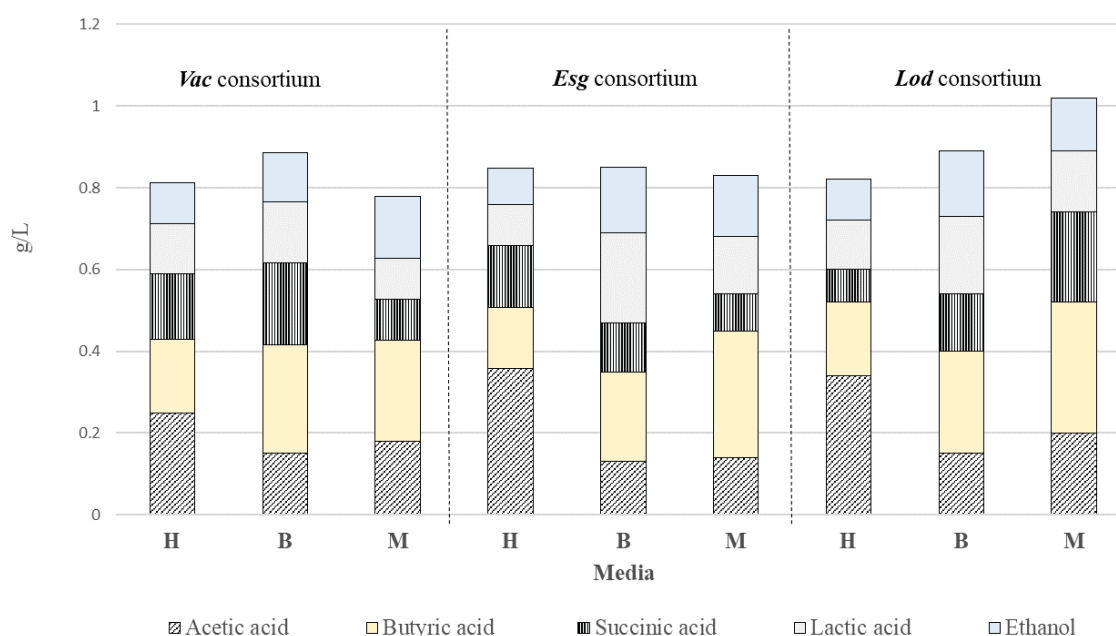
On the other hand, in the production of bioH₂ other intermediate metabolites are also produced. According to YIN and WANG, (2019); KESKIN et al. (2018), when acetic acid is the product of the reaction theoretically 4 mols of H₂ are formed, the highest theoretical yield that can be reached in dark fermentation (KESKIN et al., 2019; SIKORA et al., 2013). In the media, traces of other acids have also been found, such as butyric acid (FIGURE 14), however, when it is generated; the H₂ yields are lower due to this stoichiometric where only two moles of H₂ can be obtained. According to SIKORA et al. (2013) the formation of other non-gaseous metabolites causes a decrease in bioH₂ yields.

In addition, other compounds such as succinic, propionic and lactic acid can be generated. In this work, the production of succinic and lactic acid can be explained by the presence of bacteria of the genera *Clostridium* and *Lactobacillus*, respectively, in all consortia. However, the highest concentrations of lactic acid were detected in medium B (FIGURE 14), perhaps indicating the existence of lactic acid bacteria in the natural CPW microbiota.

The generation of propionic acid is not desirable (SAADY, 2013), which would affect the production of bioH₂. Alcohols and ketones may also be produced, however, these compounds are generally generated at the stationary phase, which could be an explanation for the presence of ethanol in the fermented media (CAPPELLETTI et al.,

2011; CHIN et al., 2003). According to HAN and SHIN (2004) the production of these compounds generates negative effects on the production of bioH₂ because the production of bioH₂ ceases. The metabolites found in all consortia indicated that fermentative processes occurred due to different metabolic pathways and reactions, but some had a negative influence on bioH₂ production (SAADY, 2013).

FIGURE 14 - Intermediate metabolic products (Acetic acid, butiric acid, succinic acid, lactic acid and ethanol) generated by the consortia *Vac*, *Esg* and *Lod* in media H, M and B.



* M: CPW sterilized; B:CPW Non-sterile; H:CPW Hydrolyzed and sterile

3.5 Conclusion

This work demonstrated the potential of CPW in bioH₂ production using the prospective consortia. However, the best performance was obtained in the hydrolyzed medium. With this potential, and knowing the optimal operating conditions, it is possible (i) to conduct experiments evaluating other modes of operation, especially continuous, where yields are generally higher; (ii) increase production scale-up and evaluate effects on production; (iii) perform mass and energy balances considering that the procedure and production conditions are established. In the three consortia studied, there was a significant presence of the genus *Clostridium*, but not necessarily its predominance, in addition, there was a significant presence of the genera *Coprococcus* and *Sporaenobacter*

in the consortia *Esg* and *Vac*, respectively. It was also observed the preference for the yeast extract as a source of nitrogen. The analysis of the organic acids allowed to establish that the fermentative process consisted of different metabolic routes in which the final products were acetic, butyric, lactic acid and some types of solvents, such as ethanol.

4. CHAPTER III (Biohydrogen production in cassava processing wastewater using microbial consortia: Kinetic parameters and optimization)

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4.1 Abstract

The use of microbial consortia is one of the strategies used to try to make viable the production of bioH₂ via dark fermentation. The objective of this study was to evaluate the use of two microbial consortia (*Vir* and *Gal*) from different Brazilian environments for bioH₂ production, using cassava processing wastewater (CPW) as the main substrate. The bioH₂ production was optimized using a Box-Behnken design with the variables (T, pH, C / N and % inoculum). Subsequently, the kinetic parameters of the fermentation in the optimal conditions were calculated and the consortia were identified molecularly through Illumina high-throughput sequencing. The molecular analyses showed that these consortia consisted of bacteria belonging mainly to four families: Porphyromonadaceae, Clostridiaceae, Ruminococcaceae, and Enterococcaceae. The quadratic models had R² regression coefficients of 92.55% and 83.7% for *Vir* and *Gal*, respectively. It was found that kinetic parameters such as specific growth rate (μ), bioH₂ productivity, and substrate consumption rate were higher in hydrolyzed and sterilized medium. The relative abundance of microbial families varies as fermentation progresses. In both consortia, Clostridiaceae reached the maximum relative abundance in the media between 16 and 24 h, interval in which approximately 90% of the bioH₂ is generated.

Key words: Optimization, bioH₂, Consortia, CPW, Kinetics.

4.2 Introduction

In the last decades, great efforts on the development of technologies to produce renewable energies and the substitution of fossil fuels have been made. However, 87% of the global energy consumption comes from non-renewable sources (PREETHI et al., 2019). Besides the drawbacks on making our energy matrix greener, the fast population growth and consequent increase on energy demand will make this scenario even more challenging. Between 2000 and 2014, for example, the per capita energy consumption increased 31% (WORLDBANK, 2019). The use of fossil fuels results in economic instability (very few countries control major petrol reserves), decrease of quality of air (ABIÁN et al., 2019) and increase of atmospheric CO₂, which is related to global warming (RAMBABU et al., 2019). The search for alternative renewable fuels with increased technical and economic viability is a priority for a more sustainable world.

Hydrogen is envisioned as the main energy vector (CHENG et al., 2019) and the main source of energy (MIRANDA, 2018) of the near future: it can be safely produced, stored, transported and transformed into different types of energy. In the transportation sector, electrochemical cells have revolutionized hydrogen technology, allowing its conversion to electricity through electrochemical reactions (RIEVAJ et al., 2019). Thus, until April 2018 8000 cars with electrochemical cells were registered worldwide (NAGARAJAN et al., 2019). These cells are also being adapted for the aeronautical sector, where they provide 8 to 10 times more autonomy and shorter refueling times in comparison to traditional fuels (BRUCE et al., 2018).

Renewable hydrogen can be produced from the dissociation of the water molecule (Thermolysis, Electrolysis and Photolysis), biological processes (Bio-photolysis, Dark Fermentation, Photo Fermentation) and thermochemical processing of biomass (Pyrolysis, Gasification, Combustion, Liquefaction) (CHENG et al. 2019; NIKOLAIDIS and POULLIKKAS, 2017). Nowadays, approximately 500 billion m³ of hydrogen are produced per year, with an annual increase of approximately 10% (KHAN et al., 2017). However, 95% of the hydrogen produced in the world comes from fossil natural gas (CHI and YU, 2018) and approx. 5% from the electrolysis of water (EL-EMAM and OZCAN,

2019; RIEVAJ et al., 2019). Although, both methods require very high energy input (NIKOLAIDIS and POULLIKKAS, 2017).

A promising alternative is the biological production of hydrogen by dark fermentation, in which organic matter (such as liquid and solid wastes) is consumed by microorganisms with simultaneous hydrogen production (“bioH₂”). Different effluents have been studied for the production of bioH₂ using pure strains or microbial consortia (SYDNEY et al., 2014; TORQUATO et al., 2017; IMACULADA et al., 2016; DAVILA-VAZQUEZ et al., 2009). However, little attention has been given to cassava processing water (CPW).

CPW is an effluent generated during cassava processing at an approx. rate of 300-600 L of CPW/ton (DE CARVALHO et al., 2018; SILVA et al., 2014). The CPW is source of macro and micronutrients, contains high organic load, which would justify its use as a possible substrate for bioH₂ production (MARTINEZ-BURGOS et al., 2019). In 2017, approximately 290 million tons of cassava were produced worldwide. The main producers are Nigeria, the Democratic Republic of Congo, Thailand, Indonesia, Brazil, Ghana, Angola, Cambodia, Vietnam, and Mozambique. Accounting for 75% of global production (FAOSTAT, 2019).

Previous research used CPW as substrate for hydrogen production via dark fermentation mostly focused on process optimization. Common optimized parameters are C/N (SREETHAWONG et al., 2010), temperature (HASSAN et al., 2015), % inoculum (FLECK et al., 2017), addition of metal ions (TRCHOUNIAN et al., 2016), enzymatic and physical treatments of the substrate (LEAÑO and BABEL, 2012). However, additional studies are still necessary to complement the existing research objectifying the calculation of the different kinetic parameters and thus understand each of the phenomena immersed in the production of bioH₂ via dark fermentation using microbial consortia. This would facilitate the staggering processes and the design of the process lines and their different stages.

In this work two microbial consortia (*Vir* and *Gal*) and CPW were identified and used for bioH₂ production, since they were new consortia, the first strategy was optimized the variables, pH, T, C/N, % inoculum. Besides, CPW underwent different types of treatments (hydrolyzed, sterilized and non-sterilizing), aiming to improve productivity and to identify if these treatments are necessary or not. Subsequently the kinetic parameters in both consortia and the variation in consortia composition during

fermentation were studied. These data would facilitate the design and scaling-up of bioH₂ production facility.

4.3 Materials and methods

4.3.1 Consortia sampling

Two samples from different Brazilian environments (TABLE 10) were collected and thermally treated (95 ± 2 °C/ 15 min) aiming to eliminate methanogens and sulfate-reducing bacteria (WONG et al., 2014). The consortia were then cultivated under anaerobiosis. The anaerobic environment was created following the technique described by Balch (BALCH et al., 1979). The removal of the oxygen from the medium was by boiling (100-105 °C) the medium under anoxic conditions (argon atmosphere). The redox potential of the medium was reduced adding NaHCO₃ (1.0 g/L) and Cysteine-HCl (0.5 g/L) at 85 °C and 65 °C, 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. The maintenance and conservation of the consortia were carried out by weekly transfer to fresh culture media. The inoculum culture medium was composed of: CPW, yeast extract (2.7 g/L), K₂ HPO₄ (2.4 g/L) and KH₂ PO₄ (1.8 g/L).

TABLE 11 - Origins of bacterial consortia in Brazilian environments.

Name	Origin	Location
<i>Gal</i>	Chicken manure	(DMM)-25.549741, -49,224515
<i>Vir</i>	Vinasse effluent	(DMM)-22.668714,-48.539922

4.3.2 Experimental design and statistical analyses

The substrate used for the production of bioH₂ was a CPW kindly provided by the company Amidos São João Ltda. (Nova Andradina, Mato Grosso do Sul, Brazil). The effluent was maintained at -20 °C until use.

The physicochemical characterization of CPW is presented in TABLE 11. The sugars content was determined by High Performance Liquid Chromatography (HPLC). Anions and cations were analyzed by ion chromatography (761 Compact IC, Metrohm AG). The columns used were Metrosep A Supp 5 - 250/4.0 and Metrosep C 3250/4.0 for anions and cations respectively. In the case of anions the mobile phase was Na₂CO₃ 3.2 mM and NaHCO₃ 1.0 mM at a flow rate of 0.7 mL/min and for the cations, HNO₃ 3.5 mM at a flow rate of 0.9 mL/min. Run times were 30 and 25 min for anions and cations, respectively. The yield (mol H₂/mol maltose) was calculated using the ideal gas equation. The determination of nitrogen, COD (chemical oxygen demand), total volatile solids, total fixed solids were done using Standard Methods for the Examination of Water and Wastewater (AMERICAN-PUBLIC-HEALTH-ASSOCIATION, 1992).

TABLE 12 - Physicochemical characteristics of CPW

Parameter	mg/L	Parameter	mg/L
Cl⁻	52.00	Na⁺	16.64
Br⁻	30.00	NH₄⁺	61.53
NO₃⁻	4.00	K⁺	435.75
HPO₄²⁻	47.30	Mg²⁺	43.08
SO₄²⁻	63.77	Ca²⁺	86.49
COD (mg O₂/L)	8488 ± 363	Total nitrogen (Kjeldahl)	180
pH	5.5	Volatile solids	3500±500
Maltose	1300	Fixed solids	1900±400
Glucose*	600		
Maltose*	1500	Total solids	5400±400

*Only hydrolysed CPW

4.3.3 Selection of the nitrogen source

Three nitrogen sources, yeast extract, urea, and ammonium sulphate, were evaluated at a C/N ratio of 3.3(moles) (CHENLIN and HERBERT, 2007). The experiment was done at 35°C, pH 7.0 and inoculum rate 20%. The pH of the media was adjusted with 1.0 M NaOH and 1.0 M HCl solutions. A one-way ANOVA test ($p \leq 0.1$) was performed for the selection of the nitrogen source with Fisher's posterior test. The CPW, besides being supplemented with the nitrogen source, was also supplemented with 2.4 g/L K₂HPO₄ and 1.8 g/L KH₂PO₄ (CAPPELLETTI et al., 2011).

4.3.4 Optimization

Five input variables were evaluated, in a Plackett-Burman (PB) design: four quantitative, pH (4-9), T (30 - 42°C), C/N (1-10), % inoculum (0%-20%) (2-5x10⁶ CFU/mL), and one qualitative (hydrolysis or not of CPW) . A Pareto Chart was used to determine the significance of effects. Two PB design with 12 experimental runs, one for each consortium, were made. Hydrolysis of CPW was performed using 1% (v/v) HCl at 121 °C/ 1 h. These variables affect the production of bioH₂ (YIN and WANG, 2019)

Once the significant parameters were defined, the variables selected for each consortium were optimized using a Box-Behnken (BB) planning with four factors and three central points. The a quadratic model, presented in equation 1, was adjusted to the results:

$$H2 = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i X_i + \sum_{i=1}^k K - 1 + \sum_{j=1+i}^k \beta_{ij} X_i X_j \quad \text{Equation 1}$$

Where β_0 , β_i , β_{ii} and β_{ij} are the constant, linear, squared and interaction coefficients of the input parameters, respectively; the index (i = 1-4), indicates the four independent variables (X_1 : temperature; X_2 : pH; X_3 : inoculum ratio; X_4 : C/N ratio), and H₂ is the accumulated production of bioH₂. All statistical analyses and surface responses were performed using the software Minitab (15). The non-significant terms of the models were excluded.

Initially the optimization of the bioH₂ production was done using the sterile CPW, after which the optimized variables were used to evaluate the bioH₂ production using S-medium, HS medium (hydrolyzed and sterile CPW) and R medium (non-sterile CPW). A control (Co) was made which was neither sterilized nor inoculated.

4.3.5 Microbial kinetic profile

Samples of 200 µL of the fermented media were collected periodically (0h, 8h, 16h, 24h, 36h and 48h), diluted and plated on MRS (De Man, Rogosa and Sharpe medium), using the Pour Plate method. The samples were anaerobically incubated at the optimum temperatures determined for each consortium, in GasPak jars with

ANAEROBAC kit during 24 h. The kinetic parameters were calculated using equations 2, 3 and 4.

$$\frac{dN}{dt} = \mu N \quad \text{Equation 2}$$

$$g = \frac{0.693}{\mu} \quad \text{Equation 3}$$

$$k = \frac{1}{g} \quad \text{Equation 4}$$

Where, N: is the number of cells / colonies (CFU / mL); t: time (h); μ : specific growth rate (h^{-1}); g: generation time; k: growth rate (generations h^{-1}).

4.3.6 Kinetic profile of bioH₂ production

Biogas production was periodically analyzed (0h, 8h, 16h, 24h, 36h and 48h). The gas produced was collected in glass syringes (5 mL and 100 mL) and analyzed on a gas chromatograph 490 Micro GC System (Agilent), equipped with two columns (Molsieve 5Å and PoraPLOT U) and a thermal conductivity detector (μ TCD). In the Molsieve 5Å, the injection temperature was 110°C, the injection time 20 ms, column temperature 90 °C, and initial pressure 190 kPa. In column PoraPLOT U (PPU), the injection temperature was 110°C, column temperature 90°C and initial pressure 150 kPa. The peak retention time for H₂ was 0.4 min (Ms5A) and for CO₂ and CH₄ were 0.405 and 0.425 respectively in the PPU. The time of each run was 1.2 min. The mobile phase used was argon with a purity of 99.999%. The productivity of bioH₂ and some organic acids was calculated using **Equation 5** in the exponential phase, and the yield of bioH₂ (mol H₂ / mol source carbon) was calculated using the ideal gas equation.

$$q = \frac{dP}{dt} \quad \text{Equation 5}$$

Where P is the concentration of the organic acids (g/L) generated in the fermentation.

4.3.7 Kinetic profile of organic compounds production

The organic compounds produced in the fermentative process were periodically analyzed (0h, 8h, 16h, 24h, 36h and 48h), using HPLC. Before injecting, samples (1.0 mL) were centrifuged at 6000 rpm (10 min) and subsequently microfiltered on cellulose acetate membranes (0.22 μ m). Analyses were done using a Agilent 1260 Infinity Quaternary liquid chromatograph, with RI detector and a Hi-Plex-H column. Detector

and column temperatures were 50 °C and 60 °C, respectively. The mobile phase was 5mM H₂SO₄ at a flow rate of 0.6mL/min and the injection volume was 10 µL. Standard curves were constructed to quantify sugars, acids and alcohols (concentrations 0.01g/L-1.5g/L).

4.3.8 Kinetic metagenomic profile

Samples of 700 µL of fermented HS medium were collected periodically (0h, 8h, 16h, 24h, and 48h) from each consortium (10 metagenomic profiles) and analyzed later according to the following protocol

4.3.8.1 Molecular identification of consortia

For each sample, 1000 µL of consortia culture were centrifuged at 12,000 x g for 1 min. The cell pellet was resuspended in 500 µL Tris-EDTA, homogenized with 10 µL of lysozyme solution (20 mg/mL) (Sigma Aldrich, Arklow, Ireland) and incubated at 30°C for 60 minutes. Then, 50 µL of SDS 10% (w/v) and 10 µL of proteinase K solution 20 mg/mL (Sigma Aldrich, Arklow, Ireland) were added to the lysis solution, followed by homogenization and incubation at 60 °C for 60 min. To this mixture, 150 µL of phenol-chloroform (25:24) was added, homogenized by inversion, and centrifuged at 12,000 x g for 5 min. The supernatant was removed, and the DNA was precipitated with 3x (v/v) absolute ethanol. Pellets were washed with 80% ethanol, dried and resuspended in ultrapure water. Total DNA was quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA).

4.3.8.2 Illumina high-throughput sequencing

The V4 variable region of the 16S rRNA gene (bases 515 to 806) was amplified from the total DNA extracted samples according to the Illumina 16S metagenomics sequencing library protocol. DNA was amplified with specific primers for the V3 region containing complementary adaptors for the Illumina platform (CAPORASO et al., 2012) using KlenTAQ polymerase (Sigma Aldrich, Arklow, Ireland). Bar-coded amplicons were generated by PCR under the following conditions: 95 °C for 3 min, followed by 18 cycles at 95 °C for the 30s, annealing at 50 °C for 30s, extension at 68 °C for 60s, final

extension at 68 °C for 10 min and hold at 10 °C. Samples were sequenced in the MiSeq platform using the 500 V2 kits, following standard Illumina protocols.

4.3.8.3 Bioinformatics and data analysis

Data generated by the sequencing went through a rigorous quality system that involved: (I) identification and removal of sequences containing more than one ambiguous base (N); (II) evaluation of the presence and complementarity of primers and barcodes sequences. Chimeric sequences detection, removal of noises from pre-cluster and taxonomic attribution were also performed using standard parameters of QIIME software package, version 1.9.0. By applying the UCLUST method (EDGAR, 2010), sequences presenting identity above 97% were considered the same operational taxonomic units (OTUs) according to the SILVA database (QUAST et al., 2013).

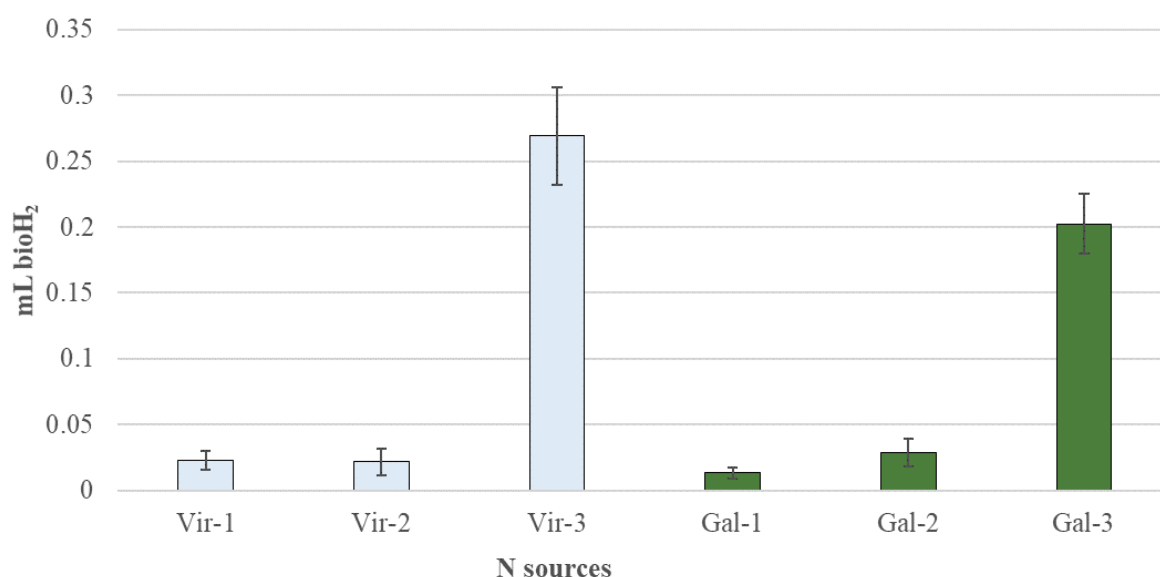
4.4 Results and Discussion

4.4.1 Selection of the nitrogen source

The nitrogen sources directly influenced the production of bioH₂ with the *Gal* and *Vir* consortia, as shown by the variance analysis. There were significant differences in the amounts produced (p-value =0.002). The volumes of bioH₂ produced are shown in FIGURE 15. In both consortia, the highest volumes were obtained with yeast extract. The volumes of bioH₂ produced with CH₄N₂O and (NH₄)₂SO₄ in the *Gal* consortium only represent 15 and 7% respectively of the volume generated with the yeast extract, while in the consortium *Vir* the volumes generated with CH₄N₂O and (NH₄)₂SO₄ represent approximately 11% of what was generated with the yeast extract. The behavior can be explained because yeast extract, is not a simple N source: it contains different compounds, from amino acids (FERRER et al., 2004; PODPORA et al. 2016), vitamins (B1, B2, B5, B6, B8, B9, B12) (FELIX et al., 2019) and minerals (Na, Ca, Mg, K, P, Fe, Cu, Mn, Zn, Si, Co, B, Mo) (FELIX et al. 2019; ZAREIA et al. 2016). These compounds stimulate the growth of microorganisms and directly the production of bioH₂ (HAKOBYAN et al., 2012a). Vitamins could also affect the physiology and metabolism of microorganisms (YILMAZ and WALHOUT, 2014). The presence of minerals such as Mg, Ni, Fe, Cu, Mo also interfere in the production of bioH₂ (BAO et al., 2013; HAKOBYAN et al. 2012) since they are enzymatic cofactors of hydrogenases (LU et al., 2013; ÖZGÜR et al.,

2010). It means that the minerals can alter the metabolic routes (ATIQUUEZZAMAN et al., 2018).

FIGURE 15 - BioH₂ produced from the different sources of nitrogen.



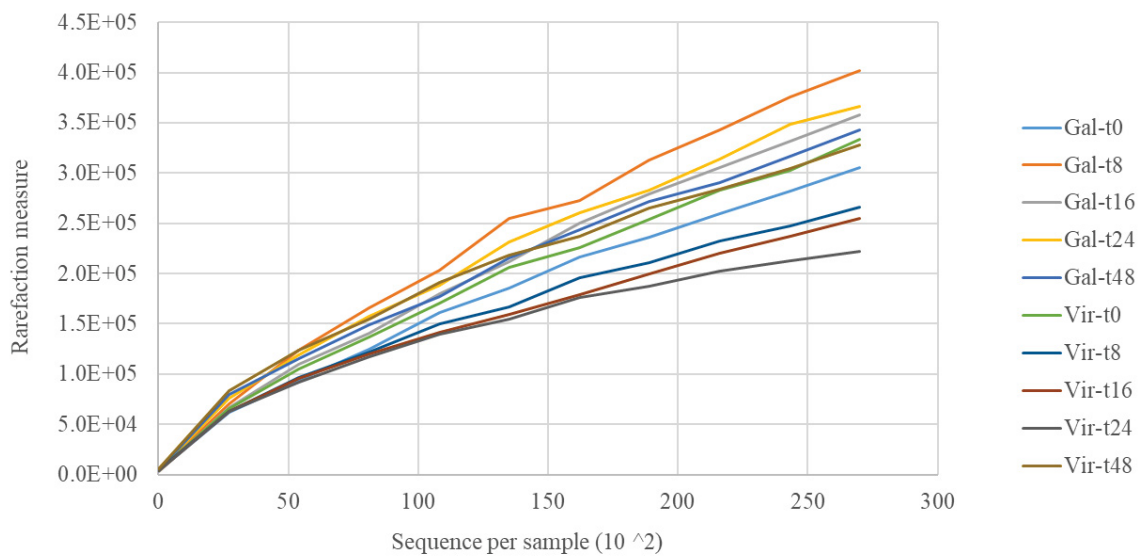
* Sources of N: 1: Urea, 2: Ammonium sulphate, 3: Yeast extract

The drawback of the use of N sources as CH₄N₂O (Urea) and (NH₄)₂SO₄ is that they need to be supplemented with other compounds, as realized by (AMORIN et al., 2014; CAPPELLETTI et al., 2011). According to (BAO et al., 2013), when salts were used as an N source, it was necessary to supplement the medium with traces of minerals and amino acids. Urea is the ideal N source, because in addition to having the highest N content (46%), it has the lowest cost. However, urea releases ammonia, which is toxic to some bacteria (MÜLLER et al., 2006). Besides, ammonia released by urea can also negatively affect the metabolic pathways of bioH₂ (GUWY et al., 2011). Notwithstanding, concentrations of up to 7.8 g L⁻¹ of ammonia can be tolerated when the production process is continuous (LIU and REN, 2008). PAN et al. (2013) also concluded that high concentrations of ammonia negatively affect bioH₂ production, finding that concentrations above 3.5g L⁻¹ decreased bioH₂ production. According to SALERNO et al. (2006) concentrations above 2 g.L⁻¹ of ammonia in batch processes significantly decrease bioH₂ production. The volume of bioH₂ produced in this work, with Urea and (NH₄)₂SO₄ showed no significant difference ($p \leq 0.05$), p-value 0.194 and 0.894 for *Gal* and *Vir*, respectively. HAKOBYAN et al. (2012), also concluded that yeast extract is an effective source of nitrogen, which improves the yield of bioH₂.

4.4.2 Molecular identification of *Gal* and *Vir* consortia

The rarefaction curves were calculated for the two consortia at different times (FIGURE 16) which reached approximately 27,000 sequences. This indicates that the sequencing is sufficient to encompass an acceptable number of the bacterial species present in each consortium.

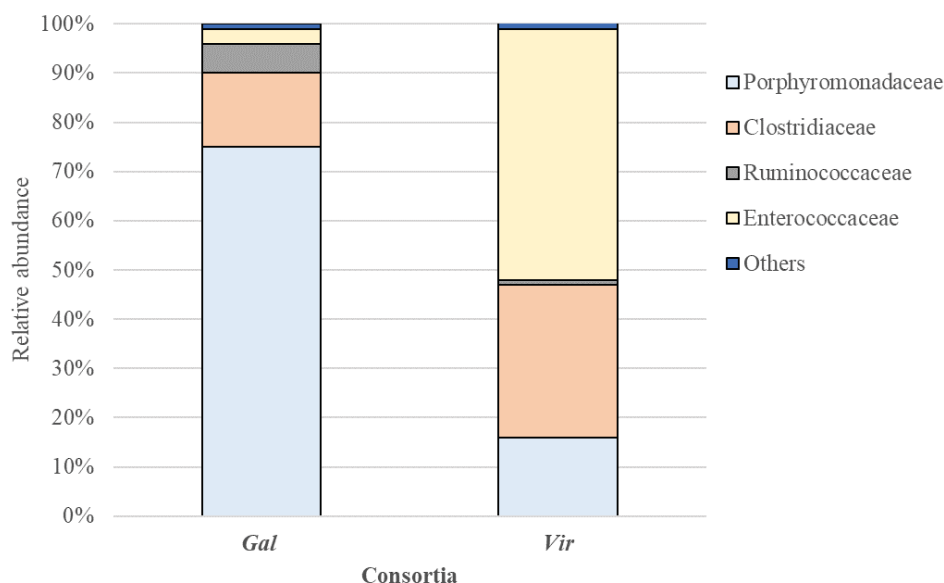
FIGURE 16 - Rarefaction curve for the bacterial community Gal and Vir consortia (times 0, 8, 16, 24 and 48h).



The molecular analyses of metagenomics allowed to establish that the consortia are composed of 5 main microbial families.

The **Vir** consortium (FIGURE 17) is composed of families Enterococcaceae (51%), Clostridiaceae (31%), Porphyromonadaceae (16%) and Ruminococcaceae (1%). While the Gal consortium is mainly composed of Porphyromonadaceae (75%), Clostridiaceae (15%), Ruminococcaceae (6%) and Enterococcaceae (3%) (FIGURE 17).

FIGURE 17 - Microbial composition for the *Gal* and *Vir* consortia. Genetic analysis based on the V4 region of the 16s rDNA gene.



The consortia have four common and predominant microbial families (Enterococcus, *Clostridiaceae*, Porphyromonadaceae, and Ruminococcaceae). Members of the Clostridiaceae family have already been found in other bioH₂ producing bacterial consortia (SYDNEY et al., 2018). GOUD et al. (2017) also found organisms belonging to the Clostridiaceae family in their bioH₂ production work using bacterial consortia in India. Besides, CARILLO et al. (2012) found an abundance of *Clostridium* in buffalo manure inoculum used to produce bioH₂. According to BALACHANDAR et al. (2013) *Clostridium* are the most studied organisms for the production of bioH₂.

On the other hand, Enterococcaceae, which also showed significant presence, is composed of four genera: *Enterococcus*, *Melissococcus*, *Tetragenococcus*, and *Vagococcus*, which are Gram-positive bacteria, facultative anaerobic, obtain the energy of different organic molecules and can produce bioH₂ (LORY, 2014). These bacteria can be found in different environments, from sites that have a nutrient deficiency as well as in dead organisms and intestines of animals. This could be an explanation of their presence in both microbial consortia. Some species of this family have been used in the production of bioH₂, such as *Enterococcus faecium* INET (YIN and WANG, 2019). Other *Enterococcus* have been reported as affecting the production of bioH₂ by competition with bioH₂-producing bacteria (NOIKE et al. 2002; SREELA-OR et al. 2011). Another microbial family found in the consortia was Porphyromonadaceae, which consists of the following genera: *Porphyromonas*, *Butyricimonas*, *Odoribacter*, *Parabacteroides*, *Macellibacteroides*, *Paludibacter*, *Petrimonas*, *Proteiniphilum* and *Tannerella*. Some of

these contain bioH₂-producing species, such as *Butyricimonas*, which are strictly anaerobic and highly butyric and isobutyric acid producers, and in smaller amounts, generate propionic and acetic acid (SAKAMOTO, 2014). The genus *Proteiniphilum* has also been related to the production of acetic acid and bioH₂ (CARDEÑA et al., 2017; SAKAMOTO, 2014). *Parabacteroides* are also involved in the production of bioH₂ and organic acids (HIGASHIMURA et al., 2018). In fact, GADOW et al. (2016) observed that the *Parabacteroides* improved the production of bioH₂ in the process of cellulose fermentation (GADOW et al., 2016). On the other hand, CATA et al. (2011) reports that *Parabacteroides* are important producers of bioH₂ because they contain Fe-Fe hydrogenase.

Finally, the family Ruminococcaceae also showed a significant presence in the Gal consortium, being composed by the microbial genera: *Acetanaerobacterium*, *Acetivibrio*, *Anaerofilum*, *Anaerotruncus*, *Ethanoligenens*, *Faecalibacterium*, *Fastidiosipila*, *Oscillospira*, *Papillibacter*, *Sporobacter*, and *Subdoligranulum*. Many genera of this family are present in different environments, mainly rumen of animals and human feces, which would explain their presence in chicken manure (KARL-HEINZ, 2009). It is a morphologically diverse family (includes long thin rods, rods, cocci and pleomorphic forms) (KARL-HEINZ, 2009), strict anaerobes, Gram positive and negative. Some species, such as *Ruminococcus flavefaciens*, *R. albus* have the ability to produce bioH₂ (KARL-HEINZ, 2009; MILLER and WOLIN, 1995; NTAIKOU et al., 2008). In fact, according to MILLER and WOLIN (1973), *Ruminococcus albus* oxidizes glucose through the Embden-Myerhof-Parnas route to acetate, ethanol, formate, CO₂ and bioH₂.

4.4.3 Optimization of bioH₂ production

The Pareto diagrams of PB showed that, in the two consortia, the five evaluated variables were significant ($p \leq 0.05$) (Figures not shown). However, the optimization was only done for the sterilized CPW. Studies developed by other researchers also showed that these variables have a significant influence on the production of bioH₂ (YIN and WANG, 2019).

The optimizations of the bioH₂ production with the *Gal* and *Vir* consortia were done using the ranges of the independent variables that are presented in TABLE 12.

TABLE 13 - Independent variable intervals.

Variables	Assesse d ranges	Coded optimal ranges		Optimal intervals		Optimum conditios used	
		<i>Gal</i>	<i>Vir</i>	<i>Gal</i>	<i>Vir</i>	<i>Gal</i>	<i>Vir</i>
pH	4.0-8.0	-0.4 0.45	to -0.3 0.25	to 5.2 to 6.9	5.4 to 6.5	6	6
T (°C)	33 - 40	-0.37 0.45	to -0.3 0.25	to 35.5 38.3	to 6.1 to 37.7	36	37
Inoculum rate, (%)	10-18	-0.37 0.50	to -0.3 0.25	to 12.5 16.0	to 12.5 to 15	13	14
C/N	3.0-10	-0.6 0.375	to -0.3 0.25	to 4.75 to 7.8	5.45 to 7.37	5.5	6.5

The contour graphs (Figures 4A and 4B) generated by the experimental design (BB) showed similar behavior. The optimal growth conditions (T °C, pH, % inoculum rate, C/N) of the consortia were very close and some are the same, for example, the pH value. This behavior can be explained by the similarity observed in microbial populations. The optimum value of the pH variable in both consortia was 6.0. According GUWY et al. (2011) the pH ranges in the acidogenic bioreactors used to produce bioH₂ are between 5 and 6. WUST et al. (2011) generated bioH₂ and organic acids using a pH range of 6.9-7.3; not the same range, but close to the intervals established in this optimization (TABLE 12). On the other hand, Enterococcaceae that are also present in both consortia can develop at pH 5.5 (KUMAR and SIVAGURUNATHAN, 2017). According to ZHENG et al. (2014) Ruminococcaceae have the best development at pH close to neutrality. Meanwhile, VALENTÍN-REYES et al. (2018) showed that the metabolism of *Clostridium beijerinckii* (microorganism known as bioH₂ producer) decreases significantly when the pH of the medium is less than 5.5. In this sense WIEGEL et al. (2005) also concluded that the growth of Clostridiaceae is higher at pH 6.0. According to KHANAL et al. (2004) pH around 4.0 prolongs the time of the adaptation phase in the microbial growth, which directly influences the time of the fermentation process and the yield of bioH₂. According to CHOJNACKA et al. (2011) the optimum pH for the production of bioH₂ is around 5.0 and its changes in the bioreactors alter the homeostasis of the bacterial communities producing bioH₂. According to HYE et al. (2008) pH is one

of the most important variables in the dark fermentation processes, so it should be optimized as it affects the activity of the enzymes Fe-Fe hydrogenases and alters the metabolic pathway.

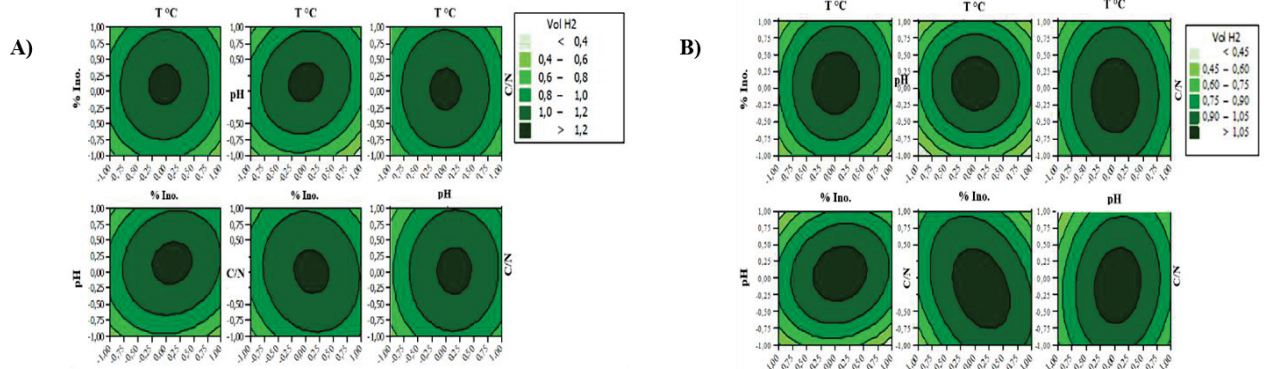
For the temperature variable, the best ones were 36 °C and 37 °C for *Gal* and *Vir*, respectively. The temperature is another variable of importance in this type of processes since it affects the growth and rate of utilization of the substrate (HYE et al., 2008) since other types of compounds can be generated (LI and FANG, 2007). The optimum temperatures found in this work are close to those reported by other authors. According to WIEGEL et al. (2005) Clostridiaceae are generally mesophilic and some have their maximum growth near 40 °C, however, there are some thermophilic species like *C. palmarium* and *C. thermobutyricum* that grow at around 65 °C. According to DAVILA-VAZQUEZ ET AL. (2009), *Enterococcus* also can grow at 37 °C, as some species of Porphyromonadaceae, Ruminococcaceae, *Ruminococcus albus*. The genera *Macellibacteroides*, *Butyricimonas*, *Paludibacter*, *Odoribacter*, *Petrimonas*, *Proteiniphilum* belonging to the family Porphyromonadaceae grow in the temperature range between 35 and 40 °C (SAKAMOTO, 2014).

Concerning the variable C/N, it is known that this is vital in the bioH₂ production processes. In this work, the optimal range was between 4.75 and 7.8. The optimal values selected were 5.5 and 6.5 for *Gal* and *Vir*, respectively, results close to those reported by other researchers (MORIMOTO et al., 2004). In the work of LIU and SHEN, (2004) they reported that bioH₂ production was maximized with a C/N of 6.7 C/N. This variable is highly sensitive in the dark fermentation processes since small changes can significantly affect the yield of bioH₂. According to ANDREANI et al. (2015), N excesses contributes to the accumulation of biomass to the detriment of bioH₂ production, reported in up to 35%. According to LIU and SHEN (2004); SREETHAWONG and CHATSIRIWATANA (2010), N excesses stimulates the production of organic acids and ethanol, decreasing the efficiency of the production of bioH₂. ELBESHBISHY et al. (2017), concluded that high concentrations of N may inhibit dark fermentation, so an optimal C/N in bioH₂ production promotes efficient fermentation (XIA et al., 2014).

Finally, for the variable % inoculum, the optimal intervals were 12.5% to 16% and 12.5% to 15% for *Gal* and *Vir*, respectively (FIGURES 18A and 18B). It was observed that an inoculum rate above 17% caused a decrease in bioH₂ production. YIN and WANG (2019) in their optimization work also found that % inoculum above 13% did not improve bioH₂ production. The % inoculum in the fermentative processes is a very important variable

that must be controlled since very high concentrations generate competition in excess between the microorganisms, which produces cell death and excess heat. However, when the inoculum% is very small, the fermentation times increase, in addition to having an increased probability of contamination (ARÉVALO and ARIAS, 2008).

FIGURE 18 - Optimized variable response surfaces. (A) *Vir* Consortium, (B) *Gal* Consortium.



Since bioH₂ is produced by different interacting microbial species, the optimal condition found is understood as the condition in which the largest number of bioH₂ producing species achieves the best performance. In this case, as already discussed, the growth of different microbial families was established between 35-40 °C, a pH near neutrality and a C/N between 5-10.

4.4.4 Mathematical models

Besides presenting the behavior of the bioH₂ production (contour graphics), BB allows to establish quadratic models to predict biohydrogen production from *Gal* and *Vir* consortia (Equations 6 and 7).

$$H_2 = 1,22 - 0,014X_1 + 0,06X_2 + 0,104X_3 + 0,015X_4 - 0,364X_1^2 - 0,30X_2^2 - 0,36X_3^2 - 0,26X_4^2 + 0,099X_1X_3 \quad \text{Equation: 6 } Vir$$

$$H_2 = 1,11 + 0,007X_1 + 0,041X_2 + 0,049X_3 - 0,045X_4 - 0,35X_1^2 - 0,28X_2^2 - 0,38X_3^2 - 0,20X_4^2 - 0,15X_2X_4 \quad \text{Equation: 7 } Gal$$

Where (i = 1-4), are the four independent variables (X₁: temperature; X₂:% inoculum; X₃:pH; X₄: C/N).

In order to validate the adjustment of the accumulated production in each model, the analysis of variance (ANOVA) was done, which is presented in TABLE 13.

TABLE 14 - Characteristics and parameters of *Vir* and *Gal* models.

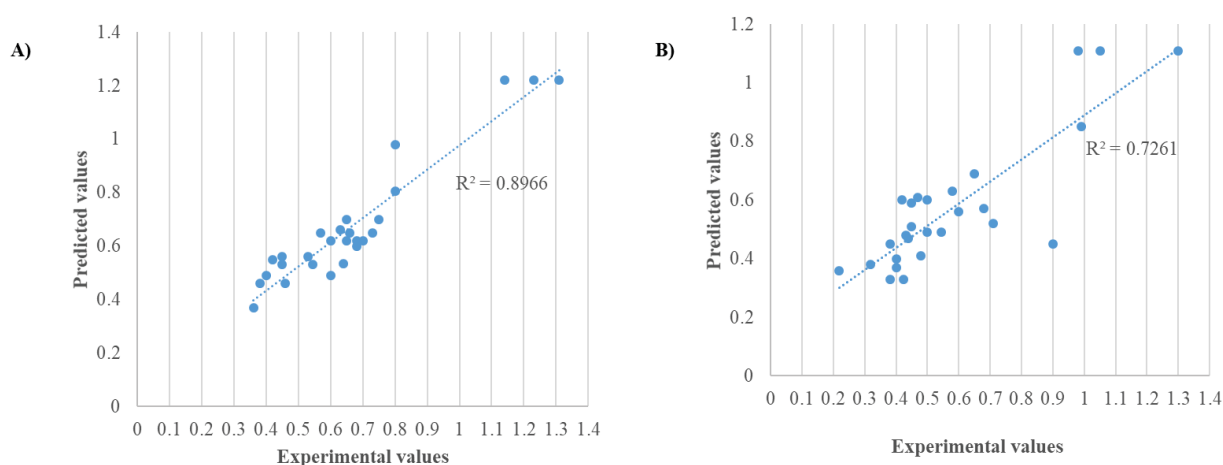
ANOVA for BioH ₂ production consortia <i>Vir</i>					ANOVA for BioH ₂ production consortia <i>Gal</i>				
Factors	Coefficient	SE-coefficient	F-value	P-value	Factors	Coefficient	SE-coefficient	F-value	P-value
Model	---	---	30.55	0.000	Model	---	---	17.37	0.000
Intercept	1.22	0.05572	23.013	0.00	Intercept	1.11	0.07464	14.871	0.000
X ₁	-0.014	0.02786	-0.523	0.610	X ₁	0.007	0.03732	0.208	0.839
X ₂	0.06	0.02786	2.183	0.050	X ₂	0.041	0.03732	1.119	0.285
X ₃	0.104	0.02786	3.604	0.004	X ₃	0.049	0.03732	1.315	0.213
X ₄	0.015	0.02786	0.568	0.580	X ₄	-0.045	0.03732	-1.221	0.245
X ₁ ²	-0.36	0.04179	-8.564	0.000	X ₁ ²	-0.35	0.05598	-6.311	0.000
X ₂ ²	-0.30	0.04179	-7.263	0.000	X ₂ ²	-0.28	0.05598	-5.127	0.000
X ₃ ²	-0.36	0.04179	-8.504	0.000	X ₃ ²	-0.38	0.05598	-6.878	0.000
X ₄ ²	-0.26	0.04179	-6.186	0.000	X ₄ ²	-0.20	0.05598	-3.596	0.004
X ₁ X ₂	0.03	0.04826	0.622	0.546	X ₁ X ₂	0.043	0.06464	0.665	0.519
X ₁ X ₃	0.099	0.04826	1.994	0.069	X ₁ X ₃	-0.0087	0.06464	-0.135	0.895
X ₁ X ₄	0.0025	0.04826	0.052	0.960	X ₁ X ₄	0.0025	0.06464	0.039	0.970
X ₂ X ₃	0.075	0.04826	1.554	0.146	X ₂ X ₃	0.085	0.06464	1.315	0.213
X ₂ X ₄	-0.06	0.04826	-1.295	0.220	X ₂ X ₄	-0.15	0.06464	-2.301	0.040
X ₃ X ₄	0.018	0.04826	0.363	0.723	X ₃ X ₄	0.058	0.06464	0.897	0.387
R ²	92.55%	Adj R ²	83.87%		R ²	87.27%	Adj R ²	72.43%	
Pred R2	60.49%	S	0.096		Pred R2	40%	S	0.12	

The determination coefficients R² showed that the models can predict the accumulated bioH₂ production with 92.55% and 87.27% efficiency. Coefficients $\geq 80\%$ indicate that the model is adequate to explain the variability of the accumulated bioH₂ production in the evaluated intervals (HYE et al., 2008). The models found are valid since the ANOVA comply with the three assumptions. (i) the residues follow the behavior of a normal distribution (Figures not shown), (ii) the residuals are completely dispersed and do not present trends (Figures not shown), and (iii) the residuals comply with the assumed homoscedasticity, that is, the variance of the error of the response variable is constant throughout the observations. Moreover, the equations are validated by the achieved values of the Durbin-Watson test (autocorrelation): 2.18 and 2.01 for *Gal* and *Vir*, respectively, as the p-values > 0.05 indicate that there is no serial autocorrelation of the errors, it means, the term perturbation of one observation is not influenced by the errors of the other (MONTGOMERY, 2004; MYERS et al., 2016). Also, it has been observed that both models can predict the behavior of the accumulated production of BioH₂. It is important to note that there was no significant difference ($p \leq 0.05$) between the experimental and predicted values in this condition. There was also no significant

difference ($p \leq 0.05$) between the volume of bioH_2 produced by the *Vir* consortium and that generated by *Gal*.

It is important to note that the overall difference between the modeled and predicted data in both models is approximately 10%; however, there are some atypical points. Nevertheless, despite the differences found between experimental and predicted values, both models present a good fit that can be observed in real and predicted graphs, which present a linear trend (PERALTA-ZAMORA and CORDEIRO, 2005) (FIGURES 19A and 19B) with R^2 of 0.89 and 0.73 for *Vir* and *Gal*, respectively.

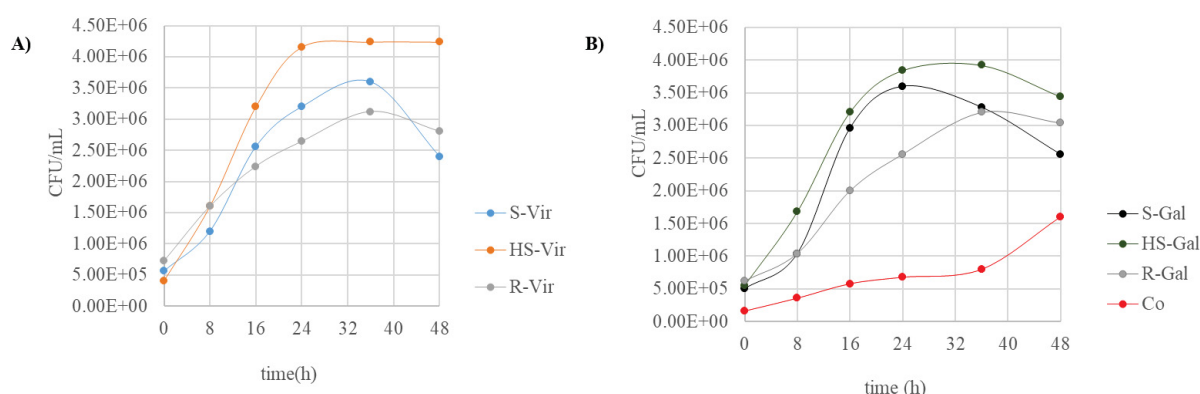
FIGURE 19 - Real values vs. predicted values for the models, *Vir* (A) and *Gal* (B).



4.4.5 Analysis of bioH_2 production under optimal conditions

BioH_2 production analyses were performed in the media (S, HS and R) to study the behavior in each medium. In the microbial kinetic study (FIGURES 20A and 20B) it was observed that the log phase for S-*Gal*, HS-*Gal*, and HS-*Vir* occurs between 8 and 24 hours, but for R-*Vir*, S-*Vir* and R-*Gal* exponential growth occurs between 8 and 36 hours. Also, it is observed that the adaptation phase is very short. This could be explained by microorganisms adaptation to the substrate (inoculum and fermentation media have the same composition) and by adequate culture conditions (pH, T and others).

FIGURE 20 - Microbial growth profile (A) *Vir* consortium, (B) *Gal* consortium.



S: CPW sterilized; R: CPW Non-sterile; HS: CPW Hydrolyzed and sterile; Co (Controle) CPW Non-sterile and without inoculating

According to LAY (2000), the optimum pH in the bioH₂ production processes is key to the decrease of the lag phase time and to increase the biogas yield. Decreasing lag phase time in the bioH₂ production processes on an industrial scale is an important factor since it is a "dead time" because the amount of bioH₂ generated in this phase is very low and sometimes zero.

On the other hand, in the mentioned graphs (FIGURES 20A and 20B), it was observed that the growth was higher in both consortia in the HS medium and relatively lower in the R medium, possibly due to the bioavailability of the substrate and the glucose produced in the hydrolysis process (0.6 g/L) because it is easier to metabolize. In fact, the specific growth rate constant (μ) in the HS medium was higher in both consortia compared to the non-hydrolyzed media (S and R). Thus, μ in the HS media was 0.06 h⁻¹ and 0.052 h⁻¹ for *Vir* and *Gal* respectively, while in the R media the constant was 0.023 h⁻¹ and 0.041 h⁻¹(TABLE 14).

TABLE 15 - Kinetic parameters BioH₂ production.

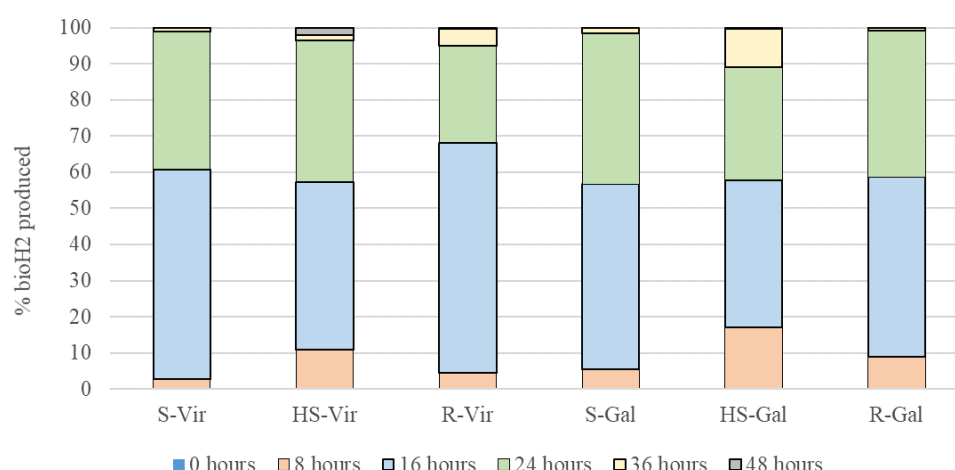
Media / Consortia	μ (h ⁻¹)	g (h)	k(h)	q				Substrate consumption (g/L/h)
				Acetic acid g/ L / h	Butiric acid g/L/h	CFU/L/h	mLH ₂ /h/L	
S- <i>Vir</i>	0.039	16.6	0.061	0.018	0.0019	85*10 ⁶	8.2	0.045
HS- <i>Vir</i>	0.06	11.6	0.086	0.043	0.0042	160*10 ⁶	21.82	0.086
R- <i>Vir</i>	0.023	29.06	0.034	0.011	0.005	54*10 ⁶	10.5	0.0625
S- <i>Gal</i>	0.048	14.36	0.069	0.022	0.0075	97*10 ⁶	12.71	0.058
HS-Gal	0.052	13.4	0.075	0.074	0.01	135*10 ⁶	16.62	0.064
R-Gal	0.041	17.26	0.057	0.015	0.00125	77*10 ⁶	12.54	0.043

As a consequence, the generation times (g) in the HS media were lower and the growth rates (k) were higher, which reflected in larger numbers of generations per unit time and directly increased bioH₂ production since the generation is linked with the production of cellular energy. In the exponential phase, the biomass productivity was higher in the HS media, followed by the S media and smaller in the R media.

In the kinetic growth profile, it can also be observed that all the consortia in 36 h of fermentation had already reached the stationary phase. In fact, HS-*Vir* and HS-Gal reached this stage in 24 hours. Theoretically, in this stage, there is no production of bioH₂, due to the production of alcohols and ketones, so it would be convenient to finalize the process, as it is another "dead time" of the dark fermentation. In fact, in FIGURE 21 it is observed that in about 24 hours approximately 95% of the bioH₂ is generated in each medium.

Between 24 and 36 hours, H-*Gal* and B-*Vir* only generated approximately 10% and 5%, respectively, of the total volume of bioH₂. It is important to note that the highest proportion of bioH₂ occurs between 16 and 24 h, the time in which the highest biomass growth occurs. This indicates that bioH₂ production is associated with biomass growth. The maximum productivity of biohydrogen was reached in the HS media (*Vir*: 21.82 mLH₂ / h / L and *Gal*: 16.62 mLH₂ / h / L), while in the non-hydrolyzed media (S and R) the productivity was in the range of 8 to 12 mLH₂ / h / L (TABLE 14).

FIGURE 21 - Kinetic profile of bioH₂ production with *Vir* and *Gal* consortia



S: CPW sterilized; R: CPW Non-sterile; HS: CPW Hydrolyzed and sterile

The first 36 h of fermentation account for approx. 98% of bioH₂ generation, a process which is much faster than the works where >95% of bioH₂ was generated in 160h (YUAN et al., 2011), 200h (FRASCARI et al., 2013) and 120h (WANG et al., 2017).

The substrate hydrolysis increased the yield (mol H₂/mol maltose) significantly ($p \leq 0.05$) between the hydrolyzed (HS) and unhydrolyzed media (R-S). Notwithstanding, there was no significant difference ($p \leq 0.05$) between the yields obtained in the R and S media. The yield reached by the *Vir* and *Gal* consortium in the HS media was 4.12 ± 0.43 and 3.80 ± 0.21 mol H₂/mol maltose, respectively. These results can be compared with those obtained by other researchers who also used disaccharide carbon source of similar molar mass. Thus, FERCHICHI et al. (2005) reported a maximum yield of 2.77 mol H₂/mol maltose, VASCONCELOS et al. (2013) reached a yield of 4.24 mol H₂/mol sucrose and CHEN and CHANG (2001) reached 3.47 mol H₂/mol sucrose, yields lower than those achieved in this study with HS media.

Concerning the unhydrolyzed media, in the S media, the yield was 2.21 ± 0.34 and 2.15 ± 0.04 mol H₂/mol maltose and in the R media was 1.82 ± 0.08 and 1.80 ± 0.04 mol H₂/mol maltose for *Vir* and *Gal* respectively. Although the yields were lower than those obtained in the HS media, they were still much more expressive than those reported by LOGAN et al. (2002), who also used disaccharides as a carbon source only reaching a yield of 1.8 mol H₂/mol sucrose.

The approximate concentration of maltose in the hydrolyzed and unhydrolyzed media was 1.6 and 1.3 g/L respectively (FIGURE 22A). Besides, hydrolysis also favored

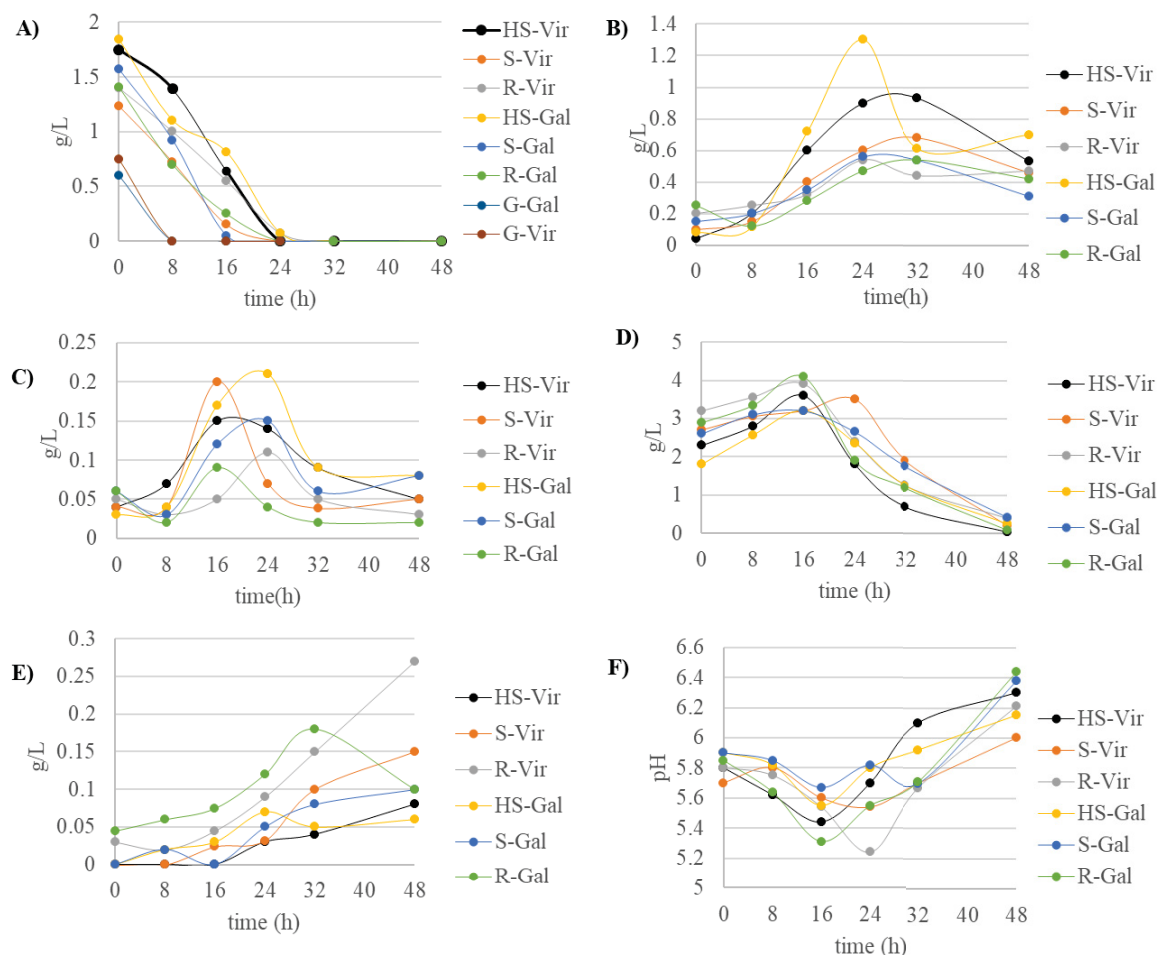
the generation of glucose in HS media (approximately 0.6 g/L) (equivalent to 1.8 g/L maltose), whereas the non-hydrolyzed medium did not present glucose. In the fermentation process, in addition to producing bioH₂ and biomass, other metabolites, such as organic acids and alcohols, have also been produced, which help to understand the reactions involved in the process (YIN and WANG, 2019). According to HASSAN et al. (2015) and WANG et al. (2017) different metabolic routes may govern dark fermentation, however the routes depend on microorganisms and conditions. The production kinetics of some organic acids and methanol are shown in FIGURES 22B, 22C, 22D, and 22E. It is observed in FIGURE 22A that the glucose (G) of the H medium is consumed in 8 hours, indicating the preference of the microorganisms for the monosaccharide. This is because simple sugars are easier to metabolize (VASCONCELOS et al. 2014). Besides, it is also observed that the total consumption of maltose occurs between 16 and 24 hours, that is in the exponential phase. At this stage, the maximum rate of substrate consumption in media HS was 0.086 and 0.064 g/L/h for *Vir* and *Gal* respectively, while in the unhydrolyzed media (S and R) was in the range 0.045 and 0.062 g/L/h.

Among the metabolites generated, acetic and butyric acid were found in high amounts (FIGURES 22B and 22C) with a sharp increase in the first 24h. The highest acetic acid concentration was reached in the HS media, approximately 0.9 and 1.3 g/L for *Vir* and *Gal*, respectively. This could be an explanation of why the higher yields of bioH₂ were obtained in these media. According to LUKAJTIS et al. (2018) and TURANBAEV and ARGUN (2018) when acetic acid is the product of the reaction 4 mol of bioH₂/mol glucose are produced, indicating that the route was of the acetate type. In the process, butyric acid was also generated; indicating that the route followed was butyrate. The acetate pathway is preferred because it regenerates the reducing equivalents, in addition to allowing the microorganism to synthesize ATP (SAADY, 2013).

It is observed that lactic acid was produced up to 16 hours of fermentation, which is due to the presence of Lactobacillales bacteria (SIKORA et al., 2013) Bifidobacteriaceae (POKUSAEVA et al., 2011) and bacteria of the genus *Leuconostoc* (MAKAROVA et al. 2006). Nonetheless, the generation of this metabolite was higher in R media (3.9 and 4.1 g/L for *Vir* and *Gal* respectively) (FIGURE 22D), indicating that the natural flora of the effluent also contains acid-lactic bacteria and also participate in the fermentation (DAMASCENO et al., 2016). This could also be an explanation of why in R media the highest concentrations of the metabolite were obtained, with substrate competition Sikora et al. (2013). The maximum production of organic acids occurs

between 16 and 24 hours, which agrees with the time range where the media reach the lower pH (FIGURE 22F). In this sense, the lowest pH were obtained in R media, in which the largest amount of lactic acid was generated. After 24 hours it is observed that the acid concentrations decrease and the carbon source is completely consumed. One likely explanation is that bacteria use lactic acid and acetic acid as the carbon source. Probably bacteria continue to generate bioH₂ after depleting carbon sources (glucose and maltose) in the stationary phase. MATSUMOTO and NISHIMURA (2007) observed a similar behavior, as they generated bioH₂, the concentrations of acetic and lactic acid decreased and at the end of the fermentation acid concentrations were practically undetected. CHOJNACKA et al. (2011) showed that the lactic acid generated in the dark fermentation was used as a second carbon source. BAGHCHEHSARAEI et al. (2009) also found that bacterial cultures continued to generate bioH₂ when the only source of carbon was lactic acid, however with low yield and substrate conversion efficiency of approximately 0.5%. According to SIKORA et al. (2013) the production of bioH₂ from organic acids (lactic and acetic) is possibly related to the genus *Clostridium* and other bacteria capable of using the butyrate route. On the other hand, ethanol was also produced in the media (FIGURE 22E), however, the highest production was observed between 24 and 48 hours, it means, when the process was in the stationary phase and some in the latency phase. According to BALACHANDAR et al. (2013); HAN and SHIN (2004), generally the stationary phase produces the largest quantities of compounds, such as ketones and alcohols, which ceases production of bioH₂. In this case, the highest concentrations of ethanol were obtained in media R (0.18 and 0.27 g / L for *Gal* and *Vir* respectively).

FIGURE 22 - . Kinetics of substrate consumption and generation of some metabolites. Substrate (A) maltose and glucose-G), (B) Acetic acid, (C) Butyric acid, (D) Lactic acid, (E) Ethanol, (F) pH.



* S: CPW sterilized; R: CPW Non-sterile; HS: CPW Hydrolyzed and sterile

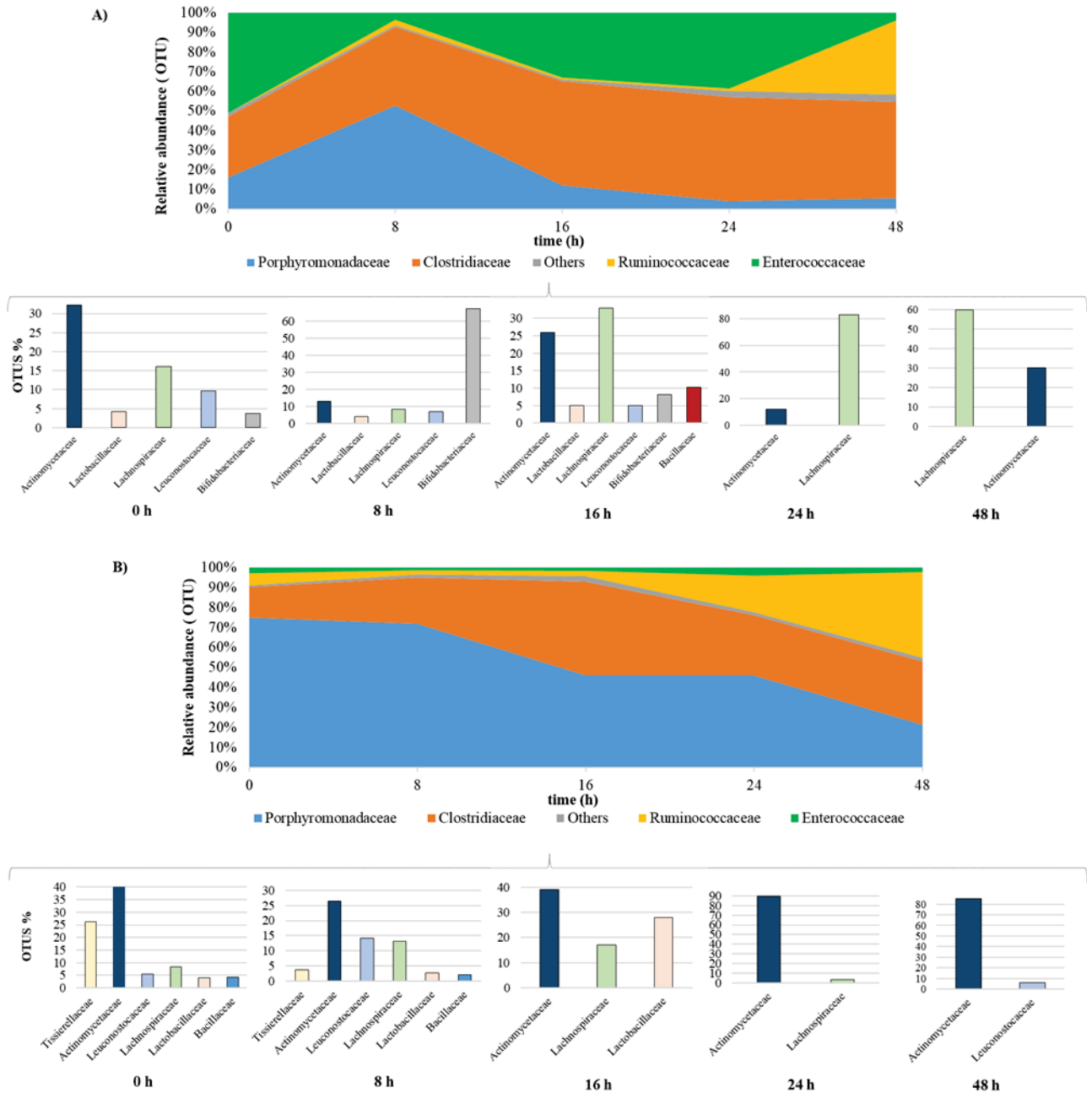
On the other hand, it was found that the relative abundance of the microbial populations in each consortium also has intrinsic kinetics, in other word; it is also altered as the fermentation proceeds (FIGURES 23A and 23B). This phenomenon assists in the understanding of the participation of each microbial community in the process. Thus, in both consortia there is a significant growth of the Clostridiaceae family between 8 and 24 h, time interval where approximately 90% of bioH₂ is produced (kinetic profile of bioH₂ production) indicating that this microbial family participates in the production of bioH₂. According to LIN et al. (2007), *Clostridium* are the dominant organisms in the production of the energetic vector. It can be observed in FIGURES 23A and 23B that the maximum relative abundance reached by Clostridiaceae in the *Vir* and *Gal* consortia were 53% and

47%, respectively. This could also be a justification for the higher yield of bioH₂ in the *Vir* consortium, although there was no statistically significant difference in the bioH₂ generated volumes by the two consortia. It was also observed that the relative abundance of the Porphyromonadaceae family at 8 h is practically constant in the *Gal* consortium, whereas in the *Vir* consortium it showed a slight increase. After 8 h its relative abundance decreases drastically in both consortia as it decreases the pH of the medium, a probable explanation is that some species belonging to this microbial family are sensitive to pH changes since they are usually microorganisms that can develop well in pH neutral which was the initial pH of the media. In fact, some researchers used extreme pH for the enrichment of bioH₂ producing bacteria (DAVILA-VAZQUEZ et al., 2008; HAWKES et al., 2002; MAINTINGUER et al., 2008). Besides, other researchers have indicated that some species of the family Porphyromonadaceae develop well at pH 7.8 (GRANADA et al., 2018). However, MOUREMBOU et al. (2016) showed that some Porphyromonadaceae only tolerate neutral pHs between 6.0 and 8.5. Moreover, pH changes in the medium in the 24 hours, as well as the substrate, are a limitation for the microbial growth, since the available carbon source of the medium (glucose and maltose) exhausts in its totality, as it was shown in the previous figures. However, some bacteria can use some organic acids as a source of carbon, including *Clostridium* (SIKORA et al., 2013). What could be an explanation of why relative abundance does not shrink as drastically as it did with the Porphyromonadaceae.

In the case of the family Ruminococcaceae, a significant increase was observed in the relative abundance between 24 and 48 h in both consortia. Probably these bacteria also can use organic acids as carbon sources.

Finally, the relative abundance of the microorganisms cataloged as others also had a significant change during the fermentation. It is observed at the beginning of the fermentation in the *Vir* consortium that predominating the following microbial families: Actinomycetaceae, Lactobacillaceae, Lachnospiraceae, Leuconostocaceae, Bifidobacteriaceae, and at the end of the fermentation only predominated Actinomycetaceae, Lachnospiraceae. On the other hand, in the *Gal* consortium, the microbial families that predominated were: Actinomycetaceae, Lactobacillaceae, Lachnospiraceae, Leuconostocaceae, Tissierellaceae, Bacillaceae and at the end of the fermentation only Actinomycetaceae and Leuconostocaceae.

FIGURE 23 - Kinetics of the relative abundance of the species of each consortium (HS medium). (A) *Vir* Consortium (B) *Gal* Consortium.



4.5 Conclusion

The effluent from cassava processing is adequate for hydrogen production. Both consortia generated significant amounts of bioH₂ in the three established media S, HS, and R. However, the yield was 50% greater in the hydrolyzed media. Both consortia are made up of similar bacterial families, which explains why the optimal conditions obtained

for pH, T, % inoculum and C/N were similar. The two mathematical models proposed for the process have the ability to predict the behavior of the response variable because the difference found between the experimental and predicted values was low, which indicates the robustness of the models. It was also found that the kinetic parameters as μ , rate of substrate consumption and bioH₂ production were higher in the hydrolyzed media, while in the non-hydrolyzed media (S and R) the values were similar. Finally, it was observed that the relative abundance of microorganisms in the consortia changes as the fermentation time advances. For example, the Clostridiaceae family reached maximum abundance at 16 h, Porphyromonadaceae decreased during fermentation and Ruminococcaceae reached the maximum relative abundance at 48 h.

5. CHAPTER IV (Development of a low-cost medium to bioH₂ production from agroindustrial effluents and scale-up of the process)

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5.1 Abstract

The use of solid and liquid residues for the production of hydrogen through dark fermentation is promising because it results in the generation of value-added products and in the reduction of the effluent's organic load. A low-cost medium using agroindustrial effluents, corn steep liquor (CSL) and cassava processing wastewater (CPW) was evaluated for hydrogen production with two microbial consortia (*Vir* and *Gal*). Four variables (agitation, purging time with Argon, % CSL and medium sterilization) were evaluated for their impact on bioH₂ production through a Plackett Burman design and only agitation was not significant ($p > 0,05$). Subsequently, the significant variables were optimized using composite central design (CCD), resulting in two mathematical models with regression coefficients $R^2 > 0.92$. The maximum yields were validated and resulted in 107 and 83.1 ml of bioH₂/g COD removed for *Vir* and *Gal*, respectively. The lower medium cost for bioH₂ production was 81.5 USD/ m³, approximately 80% more economical than some supplemented media. Finally, the scale-up of the optimal condition for bioH₂ production by *Vir* and *Gal* to 5L improved the bioH₂ production in more than 40%.

Key words: Optimization, bioH₂, Consortia, Scale up, Corn steep liquor

5.2 Introduction

Hydrogen is one of the few energy vectors that are environment friendly, because in its combustion it does not generate greenhouse gases (NIKOLAIDIS and POULLIKKAS, 2017). This is why its use as biofuel is expected to reduce up to 6Gt of annual CO₂ emissions (ACAR and DINCER, 2019) if produced from renewable sources. Currently, approx. 96% of hydrogen originates from fossil feedstocks, especially methane (KUMAR and HIMABINDU, 2019). Among the environmental-friendly technologies, the growing interest in dark fermentation applied to the use of renewable substrates such as industrial and agroindustrial effluents.

According to KUMAR et al. (2019); SWATHY et al. (2019) the theoretical maximum yield that can be achieved in bioH₂ via dark fermentation is 4 mol H₂ /mol glucose. In fact, research conducted reported yields close to the theoretical maximum. Thus, De VRIJE et al. (2002) achieved a maximum yield of 3.3 mols H₂/mol glucose. MUNRO et al. (2009) reported a maximum yield of 3.8 mols H₂/mol glucose.

However, most of the research carried out via dark fermentation hydrogen production used some type of commercial reagent to meet the nutritional deficiencies of the effluent or waste used as substrates. This fact could make H₂ production from waste impossible. For example, nitrogen and carbon source are often used for the growth of microorganisms (PREETHI et al., 2019). It is directly to produce bioH₂, since its production is linked to the redox balance during the energy production of the cells (MARTINEZ-BURGOS et al., 2019).

The use of cysteine-HCl as a reducing agent, for example, would make the production of bioH₂ on an industrial scale unfeasible (YOKOI et al., 2002). High bioH₂ yields is mostly achieved by medium supplementation, such as peptone, yeast extract, NH₄NO₃, KH₂PO₄, K₂HPO₄, MgSO₄·7H₂O, FeCl₃·6H₂O, CaCl₂ (O-THONG et al., 2010), sucrose, molasses (SYDNEY et al., 2014), CH₄N₂O, Na₂HPO₄, FeSO₄ (YOSSAN et al., 2012) and NH₄HCO₃, K₂HPO₄, MgCl₂, FeSO₄, CuSO₄ and CoCl₂ (THUNGKLIN et al., 2010). In the same studies, H₂ yield were 252 ml H₂/g starch, 7.14 mol H₂ /g sucrose, 702.5mL H₂/L-POME, and (12.77 mL H₂/g tCOD) respectively. Amino acids and minerals (Fe, Mg, Ni, Zn, Dog, Cu, Mn) have been added to improve bioH₂ production as they have the ability to alter metabolic pathways (LEE et al., 2009; SHARMA and MELKANIA, 2018a). A strategy to avoid supplementing effluents with costly reagents is to mix different types of effluents. This work proposes to produce bioH₂ in good yield without using any type of supplementation.

5.3 Materials and methods

5.3.1 Consortia and Culture Conditions

Two consortia (*Vir* and *Gal*) previously identified were used as inoculum in this research. The *Gal* consortium consisted mainly of microbial families (Porphyromonadaceae 75%, Clostridiaceae 15%, Ruminococcaceae 6%, Enterococcaceae 3% others 1%) and *Vir* was composed of (Porphyromonadaceae 16%, Clostridiaceae 31%, Ruminococcaceae 0,85%, Enterococcaceae 51% others 1,5%) (MARTINEZ-BURGOS et al., 2020).

Consortia were grown in completely anaerobic environments, following the technique described by (BALCH, et al., 1979). Oxygen was removed from the medium by boiling (100-105°C) under anoxic conditions. The medium was bubbled with argon at a flow rate of 10 mL/min for 35s. To reduce the redox potential in the medium of the inoculums, NaHCO₃ (1.0g/L) was added at 85 °C and Cysteine-HCl (0.5 g /L) at 65 ° C. The main substrate used for the media was CPW. Initially, the five consortia in the culture medium (CPW + CSL) were tested, the two best were selected and employed in this research (Results not shown).

All experiments 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 N NaOH and incubated at 37°C for 24 hours before use. The maintenance and conservation of the consortia were carried out by weekly transfer to fresh culture media. The inoculum culture medium was composed of: CPW (cassava processing waste, detailed in the next section), yeast extract (2.7 g/L), K₂ HPO₄ (2.4 g/L) and KH₂ PO₄ (1.8 g/L). The conditions used for pH, T (° C) and % inoculum in the media were optimized previously in another work. Thus, the conditions used for the *Gal* consortium were (pH: 6, T: 36°C, 13% inoculum) and *Vir* consortium (pH: 6, T: 37°C, 14% inoculum).

5.3.2 Agroindustrial effluents origin and characterization

CPW was provided by the company Amidos São Judo (Nova Andradina-Mato Grosso do Sul-Brazil) and CSL provided by the company Ingredion. The effluents were kept at -20°C prior use.

The sugars and acids were determined by high-performance liquid chromatography (HPLC). The determination of nitrogen, COD (chemical oxygen demand), total volatile solids, total fixed solids were done using Standard Methods for the Examination of Water and Wastewater (AMERICAN-PUBLIC-HEALTH-ASSOCIATION, 1992). Anions and cations were analyzed by ion chromatography (761 Compact IC, Metrohm AG). The columns used were Metrosep A Supp 5 - 250/4.0 and Metrosep C 3250/4.0 for anions and cations respectively. In the case of anions the mobile phase was Na₂CO₃ 3.2 mM e NaHCO₃ 1.0 mM at a flow rate of 0.7 mL/min and for the cations, HNO₃ 3.5 mM at a flow rate of 0.9 mL/min. Run times were 30 and 25 min for anions and cations, respectively.

5.3.3 Placket-Burman design

Four process variables were evaluated through a Placket-Burman (PB) design; three quantitative variables (CSL: 0-20%, purge time with Argon: 0-35 s at 10 ml/min, stirring speed: 0-120 rpm) and one qualitative variable (medium sterilization: yes or no). The lower limits were coded with (-1) and the upper limits with (+1). The Pareto Diagram was used to determine the significant effects of the variables on bioH₂ production. One PB design with 12 experimental runs was done for each consortium.

5.3.4 Optimization

Significant variables were optimized through composite central design (CCD) with two factors, six central points, four axial points and four hub points (14 experimental units). A quadratic model was fitted to the results. (Equation 1)

$$VH_2 = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{Equation 1}$$

Where, VH_2 is the predicted volume of bioH₂ in mL; β_0 is the constant of the models, β_i the linear coefficients; β_{ii} the quadratic coefficients and β_{ij} the interactions of

the coefficients. Statistical analyses and surface responses were performed using Minitab software version 15.

5.3.5 Kinetic profile of bioH₂ production

Biogas production was periodically analyzed (0h, 12h, 24h, 48h, 72h, 96h e 120h). The gas produced was collected in glass syringes (5 mL and 100 mL) and analyzed on a 490 Micro GC System (Agilent) equipped with two columns (Molsieve 5 Å and PoraPLOT U) and a thermal conductivity detector (μ TCD). The Molsieve 5 Å column was kept at 90 °C and initial pressure 190 kPa and the injection temperature and time were 110°C and 20 ms, respectively. The column PoraPLOT U (PPU) was maintained at 90°C and initial pressure 150 kPa, with injection temperature and time of 110°C and 20ms, respectively. The peak retention time for H₂ was 0.4 min (Ms5A) and for CO₂ and CH₄ were 0.405 and 0.425, respectively (PPU). The time of each run was 1.2 min. The mobile phase used was argon with a purity of 99.999%. The yield was calculated in (mL bioH₂/g COD removed).

5.3.6 Analyses of organic compounds

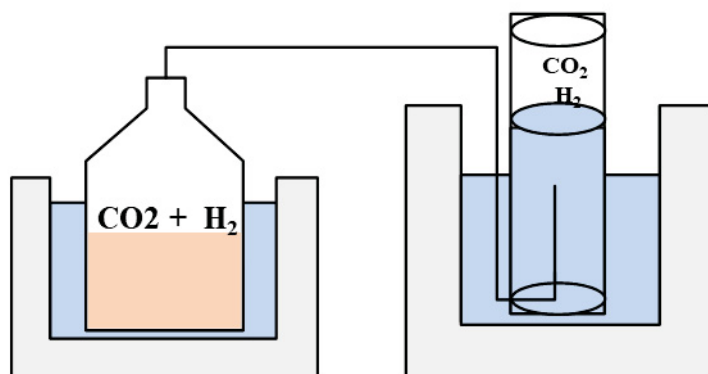
The organic compounds of the fermented medium were analyzed by HPLC. Before injecting, samples (1.5 mL) were centrifuged at 6000 rpm (10 min) and subsequently microfiltered on cellulose acetate membranes (0.22 μ m). It was used an Agilent 1260 Infinity Quaternary LC chromatograph with RI detector and Hi-Plex-H column (300*7.7 mm). Detector and column temperatures were 50 °C and 60 °C, respectively. The mobile phase was 5mM H₂SO₄ at a flow rate of 0.6 mL/min and the injection volume was 10 μ L. Standard curves were constructed to quantify sugars, acids, and alcohols (concentrations 0.01g/L-1.5g/L).

5.3.7 Fermentation Scale-Up

Scale-up was done in batch systems using 100 mL and 1000 mL bioreactors. The working volume in each bioreactor was 35% of the total volume. The culture conditions and medium composition were obtained in the optimization step. In the case of Argon purging time (Pt), the mean volume:time ratio calculated in the optimization process was

maintained constant. In the case of larger bioreactors (100, 1000 and 5000 mL), the gas was captured in another vessel (FIGURE 24) (fluid displacement method).

FIGURE 24 - Bioreactor system for bioH₂ production.



The parameters used in the scale-up are presented in TABLE 15. The variables % inoculum, T (° C) and pH were optimized in another work (MARTINEZ-BURGOS et al., 2020).. The temperature was controlled using resistances **Atman**®.

TABLE 16 - Parameters used in scale-up.

<i>Gal Consortium</i>						
Bioreactor volume (mL)	Medium volume (mL)	Inoculum volume (mL)	% CSL	Purgue time (s)	pH	T °C
16	5	0.65	6.5	17	6	37
100	31.3	4.1		109		
1000	310.3	41		1088		
<i>Vir Consortium</i>						
16	5	0.7	6.5	15	6	36
100	31.5	4.5		96		
1000	312.5	45		960		

5.4 Results and discussion

5.4.1 Physicochemical characterization

The physicochemical characterizations of CPW and CSL are presented in TABLE 16. The analyses showed that effluents are sources of macro and micronutrients, promising characteristics for their use in the production of bioH₂. In fact, ANDRADE et al. (2017) concluded that CPW is economically feasible as a substrate to obtain various value-added byproducts from the bioprocess development such as proteins, organic acids, bio-energy, etc.

On the other hand, CSL has also been considered as a promising substrate in the area of bioprocesses because it has a high content of carbon, mineral and nitrogen sources. Thus, CSL has been used in the production leukocyte interferon with *Escherichia coli* (BROWN et al., 1985), lactic acid (KRISCHKE et al., 1991; LI et al., 2006), alcohol (KUNHI et al., 1981) acetic acid (WITJITRA et al., 1995) and most recently has been used in bioenergy production (WANG et al., 2016; ZHANG et al., 2007). The organic matter and mineral content of CSL is also much higher than CPW which can be seen in COD values of each effluent. However, mixing effluents for bioH₂ production via dark fermentation showed encouraging results.

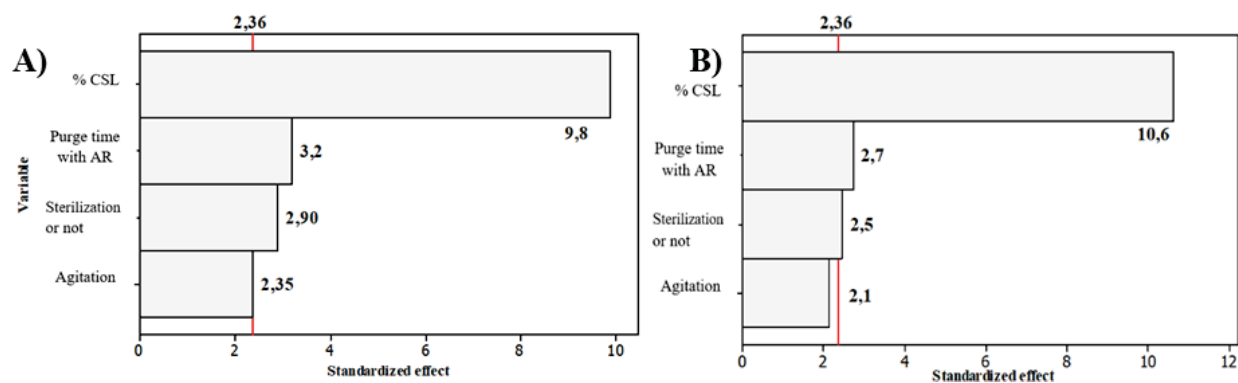
The PB design showed that among the four evaluated variables, only agitation was not significant ($p \leq 0.05$) for the consortia tested (FIGURES 25A and 25B). Excessive agitation can induce stress and destruction in microorganisms and medium oxidation. FERCHICHI et al. (2005) found that stirring of 100 rpm only improves the yield of bioH₂ in 6.0% compared to the process that was not agitated. Besides, agitated processes are more costly because demands energy input (power for impellers and turbines), specific equipment and its maintenance.

TABLE 17 - Physical chemical composition CPW and CSL.

Parameters	CPW g/L	CSL g/L
Cl ⁻	0.052	6.8
Br ⁻	0.030	ND*
NO ₃ ⁻	0.040	ND*
PO ₄ ²⁻	0.047	10.9
SO ₄ ²⁻	0.065	10.3
Na ⁺	0.016	5.05
NH ₄ ⁺	0.061	3.9
K ⁺	0.435	37
Mg ²⁺	0.043	3.5
Ca ²⁺	0.086	1.072
Total nitrogen (Kjeldahl)	0.18	15.85
Volatile solids	3.5±0.5	390.5
Fixed solids	1.9±0.4	140.5
Total solids	5.4±0.4	540
Maltose	1.3	0,15
Lactic acid	3.1	101,7
Fructose	ND	21.22
Glucose	ND	14.2
pH	5.5	4.4
COD (g O ₂ /L)	8.4 ± 0.363	124.45±0.57

*ND: not detected

On the other hand, it was found that the time of purging, the % CSL and medium sterilization were significant ($p \leq 0.05$), and thus deserves to be optimized. In this work, the medium was sterilized to ensure less influence of uncontrollable factors in the process.

FIGURE 25 – (A) Standardized effects for *Gal* consortium ($\alpha:0.05$) (B) Standardized effects for *Vir* consortium ($\alpha:0.05$).

5.4.2 Optimization

The effect of the % CSL and purge time variables on bioH₂ production by the *Vir* and *Gal* consortia were investigated using a CCD design, the planning matrix is presented in TABLE 17.

TABLE 18 - Matrix of the composite central design for *Vir* and *Gal* consortia.

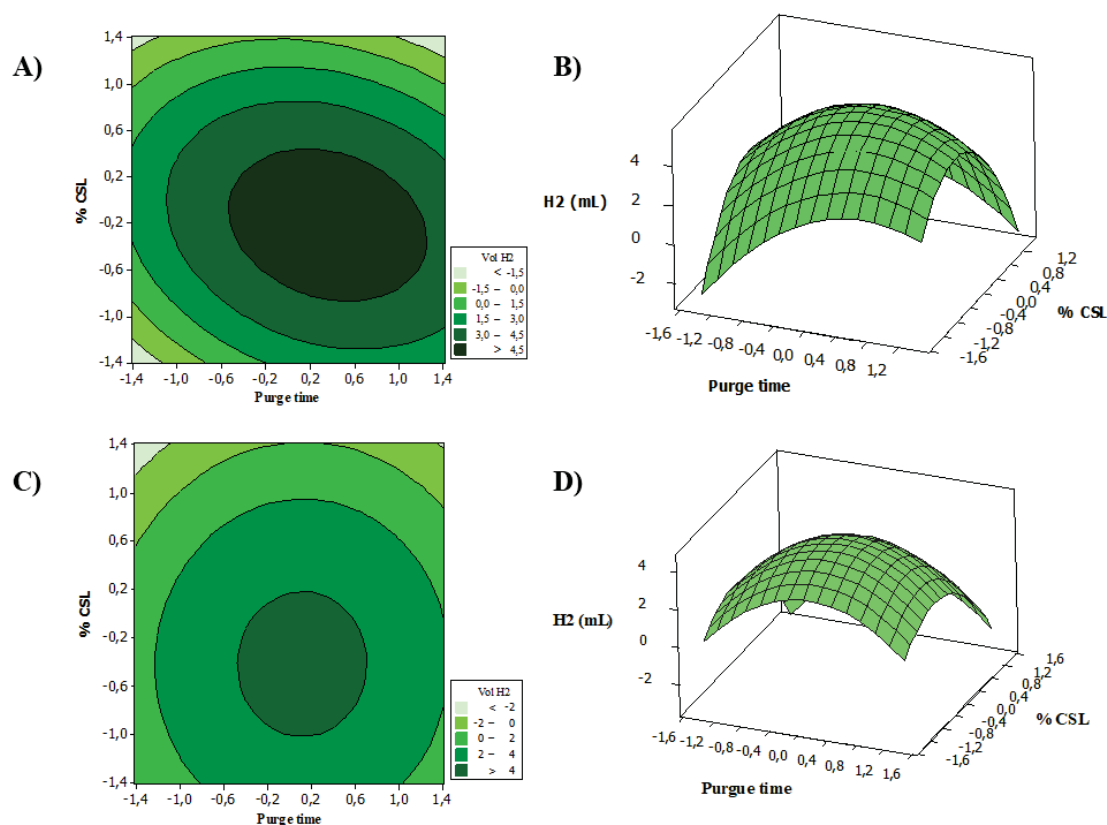
No	Purge time (s)		%CSL		Vol H ₂ mL	
	Code	Valor	Code	Valor	<i>Vir</i>	<i>Gal</i>
1	0	20	0	9.54	5,23	4,33
2	0	20	0	9.54	4,50	4,13
3	0	20	0	9.54	5,62	3,52
4	0	20	1,4	16.59	0,74	0,36
5	1,4	34	0	9.54	4,70	2,54
6	0	20	-1,4	2.49	2,31	2,71
7	-1,4	6	0	9.54	2,38	0,37
8	1	30	1	14.54	0,23	0,06
9	1	30	-1	4.54	3,24	2,81
10	0	20	0	9.54	5,04	4,48
11	-1	10	-1	4.54	0,73	3,14
12	-1	10	1	14.54	0,36	0,30
13	0	20	0	9.54	5,16	4,47
14	0	20	0	9.54	5,67	4,45

The contour and surface graphs obtained from CCD can be observed in FIGURE 26. It was also observed in the contour surfaces that higher %CSL improves bioH₂ production in both consortia. However, peak production was observed in the range of 4.54 % to 10.54 % (FIGURES 26A and 26B) and 5.04 % to 11.79 % (FIGURES 26C and 26D) for *Gal* and *Vir*, respectively. This can be explained by the fact that CSL is a source of carbon, nitrogen, amino acids (ALA, ARG, CYS, ASP, GLU, TRP, GLY, HIS, LLE, LEU, LYS, TYR, MET, PHE, PRO, SER, THR, VAL), vitamins (Biotin, Choline, Inositol, Niacin, Pantothenic Acid, Pyridoxine, Riboflavin, Thiamine) and minerals (Ca, Cu, Mn, Fe, Mg, K, Na, P, Se, Z, S) (DOMINGOS, 2009), which can participate in the different metabolic routes for the production of bioH₂ and increase the production of hydrogen.

On the other hand, it is also observed in the contour surfaces that CSL concentrations higher than 10.55% and 11.80% do not improve bioH₂ production in the *Gal* and *Vir* consortia, respectively. This can be explained by the high nitrogen content

of CSL (3.9 g/L N-NH₄ and 39.1 g/L TKN – TABLE 16). According to PAN et al. (2013) high concentrations of organic nitrogen can transform into ammonium which affects the microbial growth and metabolic pathway of bioH₂. Besides, excesses of inorganic N also inhibits bioH₂ production (SEIFERT et al., 2010; ZAMPOL and CAROLINA et al., 2015). According to HAMILTON et al. (2018), a probable explanation is the internal changes of the pH in the bacterium which would increase the energetic requirements, besides affecting the enzymatic systems mainly those that participate in the production of bioH₂, such as the hydrogenases.

FIGURE 26 - Contour and response surfaces for *Vir* and *Gal* consortia. (A) and (B): *Vir* Consortium; (C) and (D): Consortium *Gal*.



Different studies have evaluated commercial sources of nitrogen such as yeast extract, peptone, ammonium sulfate, urea, ammonium chloride, etc. with good yields; however, the use of these sources adds costs and makes bioH₂ production unviable. Furthermore, some researchers supplemented the media with other compounds such as minerals, amino acids, and vitamins as a strategy to increase bioH₂ production, further raising production costs and possibly making the process unattractive to the industrial sector. SHARMA and MELKANIA (2018c) improved bioH₂ production by 2.5-2.2 times

using concentrations between 1-10 g /L of an amino acids mix (MET, ALA, HIS, CYS, LYS). YUAN et al. (2008) and an increase of 1.5-2.9 times using L-cysteine (0.1-1.0 mM). They have also observed a synergistic effect among some amino acids (MET, ALA, HIS, CYS, LYS) and Fe₂O₃ in bioH₂ production.

Other investigators such as DING et al. (2009); LIU et al. (2010); PAN et al. (2008); SYDNEY et al. (2018) supplemented the media with reducing agents such as Cysteine- HCl. Vitamins and minerals can also improve bioH₂ production because they can also alter metabolic pathways (BAO et al., 2013; LU et al., 2013), especially the latter which are enzymatic cofactors of hydrogenases. However, these reagents are costly and their use negatively impact on process economics. In this work, significant amounts of bioH₂ were obtained without employing any commercial reagent. This is highly attractive to industries since CSL and CPW are byproducts of low to no commercial value.

On the other hand, it was observed that with Pt smaller than 14 to 16 seconds for *Vir* and *Gal* respectively significantly affects bioH₂ production. A likely explanation is that with shorter times residual oxygen concentrations could be found which affect the growth of strict anaerobic cultures and directly bioH₂ production.

The consortia consisted mainly of four bacterial families, three of which are strict anaerobes (Clostridiaceae, Porphyromonadaceae, and Ruminococcaceae) KARL-HEINZ, 2009; PANDEY et al., 2013; SAKAMOTO, 2014). Besides, the consortia also contained species of the Enterococcaceae family which are facultative anaerobes (LORY, 2014) and could withstand the presence of oxygen. These species could consume the oxygen of the environment and favor the growth of strict anaerobes.

Other bioH₂ production work has also reported oxygen purge processes with Ar, N₂, CO₂, etc. ZHANG et al. (2006) reported a purge time of the 30s for a 120 mL bioreactor, however, the authors did not present the flow rate used for comparison purposes. In another work SHI et al. (2011) reported a purging time with N₂ of 5 min for a volume of 3L medium, the fact that it has a larger volume of the medium means that a longer time is required of a purge.

5.4.3 Mathematical Models

Linear regression analysis of the experimental data from each consortium generated two-second order polynomial models (Equation 2 and Equation 3) with correlation coefficients, R², of 92.18 and 94.37 for *Gal* and *Vir*, respectively. Also, it can

be inferred that 8% and 6% of the variability of bioH₂ production is caused by external factors that were not controlled.

$$VH_2 = 4,22 + 0,31X_1 - 1,11X_2 - 1,366X_1^2 - 1,32X_2^2 \text{ (Equation 2: } \textit{Gal} \text{)}$$

$$VH_2 = 5,20 + 0,70X_1 - 0,70X_2 - 1,18X_1^2 - 2,18X_2^2 - 0,6622X_1X_2 \text{ (Equation 3: } \textit{Vir} \text{)}$$

Where: VH₂: Accumulated volume of bioH₂; X₁: Purge time (s); X₂:% CSL.

The main characteristics of the mathematical models are shown in TABLE 18. The ANOVA showed that the linear terms X₁ and X₂, quadratic and their interaction were significant ($p \leq 0.05$) for the consortium *Vir*. In the case of the *Gal*, it was found that the model is mainly governed by the quadratic terms.

TABLE 19 - Characteristics of quadratic models for *Gal* and *Vir* consortia.

<i>Gal</i> Model			<i>Vir</i> Model		
Term	Coefficient	p-value	Term	Coefficient	p-value
Intercept	4,22	0,00	Intercept	5,20	0,00
X ₁	0,31	0,201	X ₁	0,70	0,022
X ₂	-1,11	0,002	X ₂	-0,70	0,023
X ₁ ²	-1,36	0,001	X ₁ ²	-1,18	0,002
X ₂ ²	-1,32	0,001	X ₂ ²	-2,18	0,00
X ₁ X ₂	0,022	0,945	X ₁ X ₂	-0,66	0,094
Linear regression		0,04	Linear regression		0,01
Quadratic regression		0,00	Quadratic regression		0,00
R ² =92,81	Adj	Pred	R ² =94,37%	Adj	Pred
	R ² =86,645	R ² =32,13%		R ² =89,54%	R ² =71,79%

ANOVA is valid because the three assumptions are satisfied: (i) the residuals fit a normal distribution (FIGURES 27A and 27B), (ii) the residues are completely dispersed, that means, they do not present trends (FIGURES 27C and 27D), (iii) the Durbin-Watson statistic values (0.94 and 1.70 for *Gal* and *Vir*, respectively) indicated no serial autocorrelation of the errors, and (iv) the homoscedasticity was respected (the p-value of the Bartlett test were equal to 0.168 and 0.11 for *Vir* and *Gal*, respectively). The models can be considered robust because they accurately predict the production of bioH₂ with R² of 0.91 and 0.92 for *Vir* and *Gal*, respectively (FIGURE 27E and 27F). The

optimized condition was validated and showed no significant difference ($p \leq 0.05$) between experimentally produced bioH₂ volumes and those calculated with the models (TABLE 19).

FIGURE 27 - Adjustment of the residuals to a normal distribution (*Vir* consortium: **A**, *Gal* consortium: **B**); Residual dispersion diagrams (*Vir* consortium: **C**, *Gal* consortium: **D**) and actual values vs. predicted values for the models *Vir* (**E**) and *Gal* (**F**).

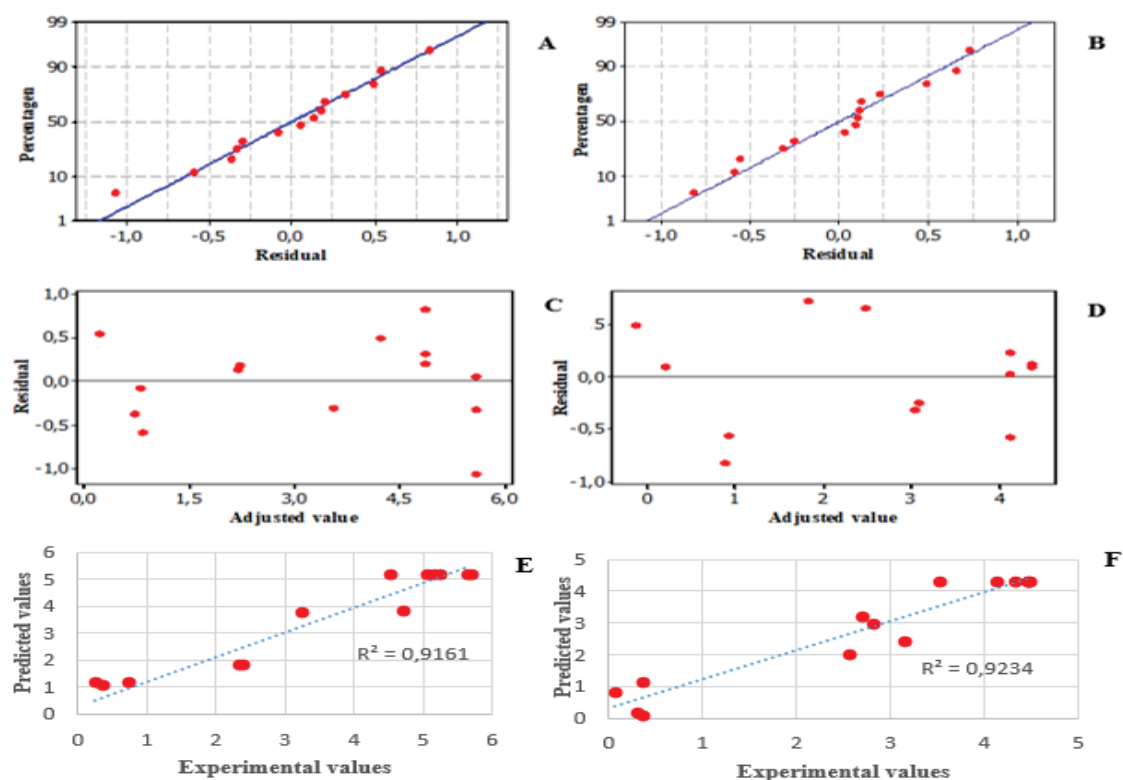


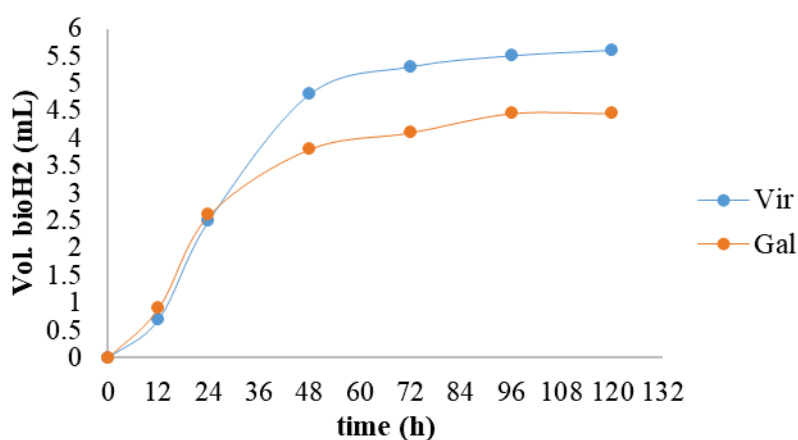
TABLE 20 - Validation of *Gal* and *Vir* models, experimental and predicted values.

Consortia	Experimental values (mL)	Modeling values (mL)	Optimal conditions according to model		Optimal intervals	
			%SCL	Time purge (s)	%SCL	Time purge (s)
<i>Gal</i>	4,1±0,48	4,32	9,54	21	4,54-10,54	15-27
<i>Vir</i>	5,15±0,39	5,3	9,54	23	5,04-11,79	15-32

5.4.4 Evaluation of bioH₂ production under optimum conditions

The fermentative production of bioH₂ was performed under previously optimized conditions: *Gal* (%CSL: 6.5; Pt:20s), *Vir* (%CSL: 6.5; Pt:20s). The accumulated production of bioH₂ by the two consortia is shown in FIGURE 28. It is observed that in both cases, at the 72 hours of fermentation, approximately 95% of bioH₂ was generated. The maximum observed cumulative production of bioH₂ were 1.1 and 0.89 L bioH₂ /L medium for *Vir* and *Gal*, respectively, which are close to those reported by other researchers. YIN and WANG (2019) obtained a maximum yield and 1.35 L bioH₂ /L medium. YIN and WANG (2016) reported maximum production as 1.16 L bioH₂/L medium. However, the media used by both researchers were supplemented with commercial reagents such as glucose, peptone, yeast extract and/or a nutrient solution of approximately 10 salts, while in this work the similar bioH₂ production was achieved with a combination of agro-industrial byproducts.

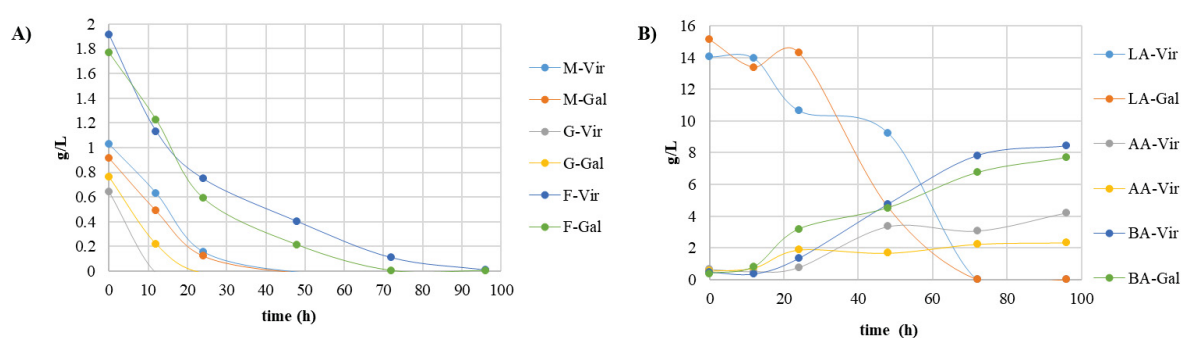
FIGURE 28 - Cumulative production of bioH₂.



During the fermentation of the medium by *Vir* and *Gal* process all major carbon sources were completely consumed with the production of bioH₂ and some volatile organic acids (FIGURE 29). It was observed that propionic acid (0.60 g/L) was generated by the consortium *Gal*, but not by *Vir*. The generation of propionic acid consumes H₂ and, thus, is not desirable (SAADY, 2013). Another metabolite produced that also affects negatively the production of bioH₂ is ethanol (SIKORA et al., 2013), which reaches concentrations of 1.37 and 1.14 g/L for *Vir* and *Gal*, respectively.

In the case of lactic acid, it is observed that it was consumed practically in its totality (FIGURE 29B). Other studies have reported that lactic acid can also be used by microorganisms such as *Clostridium sp.* as a substrate for the production of bioH₂ (SIKORA et al., 2013). Note that lactic acid was consumed in parallel with the other carbon sources (maltose, glucose, fructose). Besides, the generation of acetic and butyric acid was observed in both consortia. According to (SYDNEY et al., 2020) the production of these acids is desirable, since 2 and 4 moles of hydrogen are generated for each mole of butyric and acetic acid produced respectively. This could explain the greater volume of hydrogen obtained with the *Vir* consortium.

FIGURE 29 - (A) Carbohydrate consumption; (B) Production of organic acids.



M: maltose; G: glucose; F: fructose; LA: lactic acid; AA: Acetic acid, BA: butyric acid

In contrast, it was observed a significant decreased of approx. 50-60% in COD (from 9.3 ± 0.42 g O₂/L to 4.1 ± 0.33 g O₂/L and 4.9 ± 0.25 g O₂/L for *Vir* and *Gal*, respectively) was also observed. It is interesting to use dark fermentation in the treatment of effluents, since this reduces the organic load with the generation of products of high commercial value, such as H₂. However, additional steps will be needed to lower the COD until the legislation is met, such as recovering the generated volatile fatty acids.

The maximum yields achieved were 107 and 83.1 ml of bioH₂/g COD removed for *Vir* and *Gal* respectively. These results are superior to those reported by other studies that also used CPW as substrate. Intanoo et al. (2015) reported a maximum yield of 39.83 ml bioH₂/g COD, Lucas et al. (2015a) yielded 31.41 ml bioH₂/g COD and Reungsang et al. (2006) showed a maximum of 71.3 ml bioH₂/g COD. Besides, the productivities were 14.72 and 11.4 mL/L/ h for *Vir* and *Gal* respectively, values significantly higher than those reported by others works. Lucas et al. (2015) reported a maximum productivity of 1.51 mL / H₂ / L medium / h, in other words, 10% of the productivity achieved in this work. Reungsang et al. (2006) also achieved bioH₂ productivity similar to that reported by Lucas et al. (2015).

On the other hand, comparing the costs of raw materials for the production of media with other researchers found that some of the lowest costs were obtained in this work. REUNGSANG et al. (2006) also used CPW as substrate obtained similar costs to those exposed in this research (TABLE 20), however, their maximum yield was 20 and 34% lower than those obtained with the *Gal* and *Vir* consortia respectively.

Work developed by Marques et al. (2015) and Ozkan et al. (2011) to produce bioH₂ have been supplemented with Fe, Co, Se, K, P salts and nitrogen sources such as CH₄N₂O, and (NH₄)₂HPO₄. Whereby the costs of raw materials to produce 1 m³ of medium were high, about 707 USD and 180 respectively, (Table 6). In the case of Thanwised et al. (2012) only supplemented with FeSO₄, the cost of production is still quite high (USD 472 / m³ medium).

Additionally, in the table also estimated the cost of producing 1L H₂ (it is clear that these costs only included the prices of raw materials). Among the researches referenced the lowest cost of production was obtained in this work with the consortium *Vir* 0.15 USD / L H₂ Produced and the highest 7.85 USD/L H₂ produced (THANWISED et al., 2012). It is observed that (ZAMPOL, et al., 2015) although supplementing with different reagents the production cost of 1L of H₂ was 0.18 which is explained by the high yield, besides the low concentrations of the reagents used.

TABLE 21 - Costs of some media used for bioH₂ production.

Substrates	Reagents used	Cost medium USD	m ³	Yield (mL bioH ₂ /g COD)	Cost/L H ₂ USD	References
CPW	--	75		71.3	--	(REUNGSANG et al. 2006)
CPW	FeSO ₄	472		18.7	7.85	(THANWISED et al. 2012)
CPW	--	75		54.22	0.27	(INTANOO et al. 2014)
CPW	NH ₄ HCO ₃	167		40	--	(INTANOO et al. 2016)
Sugarcane vinasse	NiSO ₄ , FeSO ₄ FeCl ₃ , CoCl ₂ , CaCl ₂ , SeO ₂ , KH ₂ PO ₄ , K ₂ HPO ₄ , Na ₂ HPO ₄ , Vitamin B12, Aminobenzoic acid, Biotin	83		61.25	--	(ZAMPOL et al. 2014)
Sugarcane vinasse	CH ₄ N ₂ O, NiSO ₄ , FeSO ₄ , FeCl ₃ , CoCl ₂ , CaCl ₂ , SeO ₂ , KH ₂ PO ₄ , KHPO ₄ , Na ₂ HPO ₄	180		75.1	--	(MARQUES et al. 2015)
Sugarcane vinasse	NiSO ₄ , FeSO ₄ , FeCl ₃ , CoCl ₂ , CaCl ₂ , SeO ₂ KH ₂ PO ₄ , K ₂ HPO ₄ Na ₂ HPO ₄ , Vitamin B12, Biotin NaHCO ₃ , Yeast extract	284		137	0.18	(ZAMPOL et al. 2015)
Beet-pulp	NH ₄ Cl, MgSO ₄ , KCl, Na ₂ S, CaCl ₂ , (NH ₄) ₂ HPO ₄ , FeCl ₂ , CoCl ₂ , MnCl ₂ , CuCl ₂ , ZnCl ₂ , AlCl ₃ , NaMoO ₄ , H ₃ BO ₃ , NiCl ₂ , NaWO ₄ , Cysteine	707		115.6	2.44	(OZKAN et al. 2011)
POME	Fe ₂ (SO ₄) ₃ , Peptone, Na ₂ HPO ₄	188		215	0.27	(KRISHNAN et al. 2016)
Paper Mill Effluent	CuSO ₄ , NaHCO ₃ , MnSO ₄ , MgCl ₂ , CoCl ₂ , MnSO ₄ , FeSO ₄ , NiSO ₄	287		55.4	--	(VAEZ et al. 2017)
CPW and CSL	--	81.5 81.5		107 83	0.15 0.23	This work (<i>Vir</i> Consortium) This work (<i>Gal</i> Consortium)

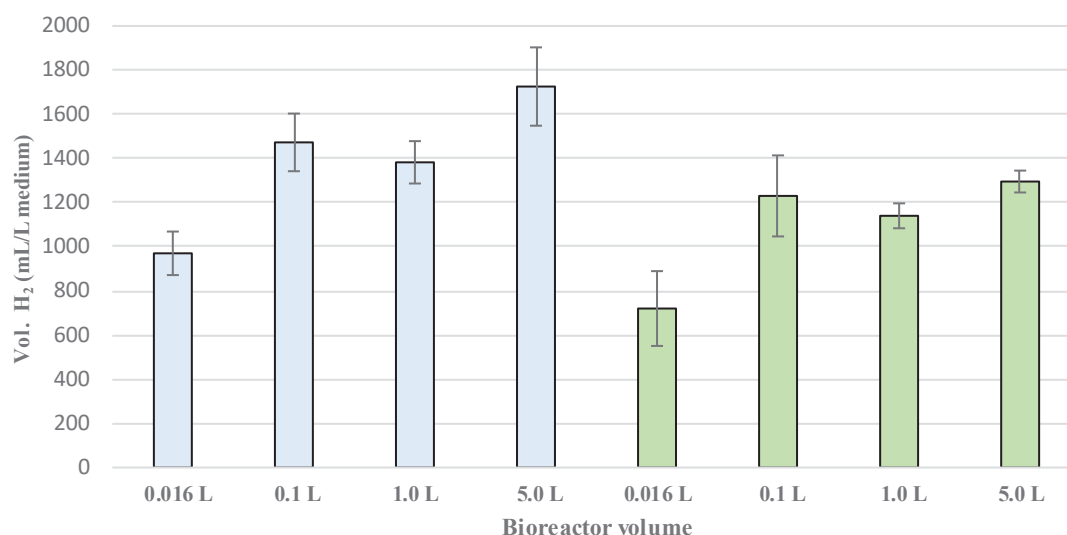
*These costs were calculated solely with the prices of raw materials and /or reagents employed.

5.4.5 Scale-up

The scaling-up of the process improved the yield of bioH₂ (Vol. bioH₂ /Vol. Medium) (FIGURE 30) by 41% and 37% for *Gal* and *Vir*, respectively. It was also observed that in the smaller bioreactors the bioH₂ concentration was approximately 35% while in the 100 mL bioreactor the concentration was approximately 45%. Comparing the volume of hydrogen produced, it was observed that on average the scaling-up improved hydrogen production by 51 and 55% for the *Gal* and *Vir* consortium respectively (FIGURE 30). It is noteworthy that the volume of hydrogen produced by the *Vir* consortium has always been greater than that produced by the *Gal* consortium.

Previous work has also shown that the scaling process increases bioH₂ productivity. CHANG et al. (2002), who scale up from 0.3L to 3L and observed a 45% increase in productivity (from 0.41 L /h/L to approx. 0.6 L/h/L). A possible explanation is that in a small reactor high pressures of about 3000 kPa can be easily achieved due to gas accumulation in the fermentation vessel, while in larger scales it was released to another container (FIGURE 24) and the pressure did not exceed 100 kPa. Internal pressure is an important parameter to be considered in bioH₂ production due to the feedback inhibition. At high partial pressure of bioH₂ the changes in metabolic pathways and flux are observed further increasing the production of lactate and solvents and decreasing hydrogen yields, while the low ones favor the H₂-friendly route of butyrate (ASLAM et al., 2018; e HALLENBECK and GHOSH, 2009; SYDNEY et al., 2014).

FIGURE 30 - Volume of bioH₂ generated at different scales.



Extrapolating bioH₂ production yields with the amount of CPW generated in Brazil in 2017 (6,795,529 m³), it is estimated that approximately 10 million m³ of H₂ could be produced in the country.

5.5 Conclusion

This work, besides showing new perspectives of bioH₂ production using only agribusiness effluents, also presents significant yields (Vir: 107 ml bioH₂ / g COD; Gal: 83.1 ml bioH₂), which can be compared to those reported in other research, where the media were supplemented with different commercial reagents. The effluents CPW and CSL are sources of different types of nutrients such as carbohydrates, minerals, and nitrogen and proved to be promising in bioprocesses, such as for the production of bioH₂. The yields obtained from bioH₂ are better than studies that used supplementation with commercial substances, which results in economic advantage.

The production of bioH₂ by mixing the effluents mentioned above is promising and important especially for companies dedicated to cassava and corn processing, as both the CPW and CSL generated in their processes negatively impact the environment and conventional treatments are expensive. Hydrogen production via dark fermentation removed approximately 50% of the organic charge. By which one could think of using mixed processes to remove the organic charge, or in addition to taking advantage of bioH₂ should also be used the other metabolites produced such as acetic and butyric acid.

6. Overall conclusion

Agro-industrial effluents are energy sources that can be harnessed or can become highly polluting wastewater when they have not been treated or used to the best of their ability. Bioenergy production from CPW and CSL under the conditions employed in this study with bacterial consortia (*Vac*, *Lod*, *Esg*, *Vir*, *Gal*) promoted a decrease in COD of approximately 50%. It is noteworthy that in this five consortia the family Clostridiaceae was found. In addition, hydrogen yields were higher than those reported in other researches, as well as the production of some organic acids such as acetic acid, butyric acid, lactic acid and others. The latter virtually disappears in the process as it is also used as an alternative carbon source. In this work, it was also found that the type of nitrogen source used for microbial growth is determinant in bioH₂ production, besides quantitative variables, such as C / N, inoculum %, pH, T (°C), time purging and the proportion of effluents, are variables that need to be optimized because their effects on bioH₂ production are significant. Also, some substrate treatments, such as acid hydrolysis and sterilization, showed a positive effect on the production of the energy vector, since the obtained yields were higher as well as the kinetic parameters. However, a technical-economic study is necessary to know the financial viability of the process. Finally, it was observed that the scaling process improved bioH₂ productivity by up to 70%.

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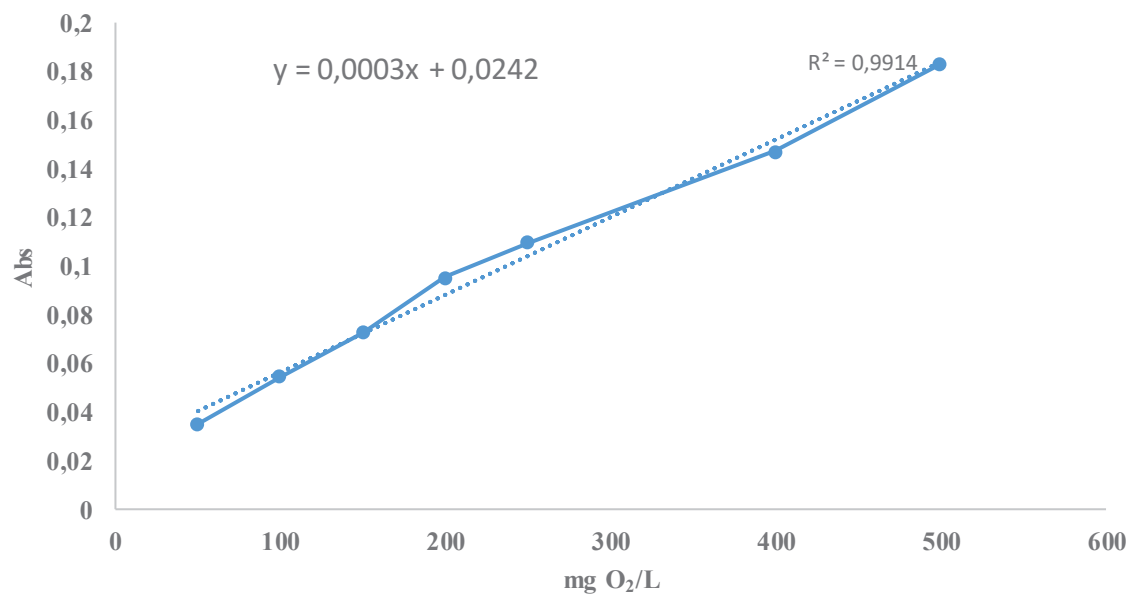
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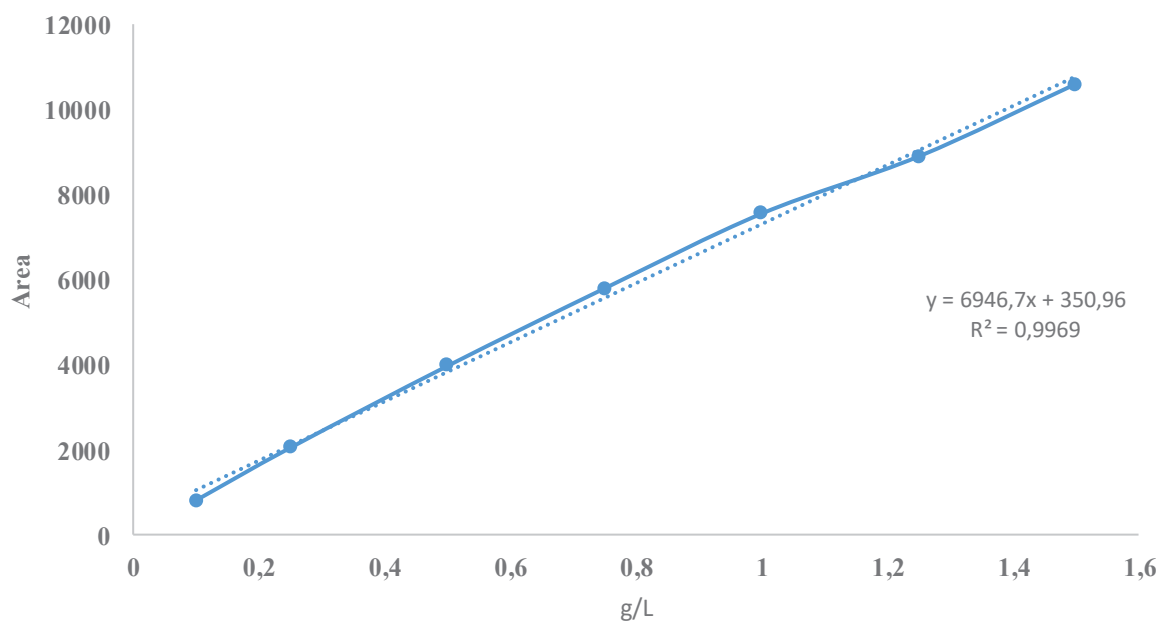
ZIARA, R. M. M.; MILLER, D. N.; SUBBIAH, J.; DVORAK, B. I. Lactate wastewater dark fermentation: The effect of temperature and initial pH on biohydrogen production and microbial community community. **International Journal of Hydrogen Energy**, v. 44, n. 2, p. 661–673, 2019. Elsevier Ltd. Disponível em: <<https://doi.org/10.1016/j.ijhydene.2018.11.045>>.

8. ANNEXES

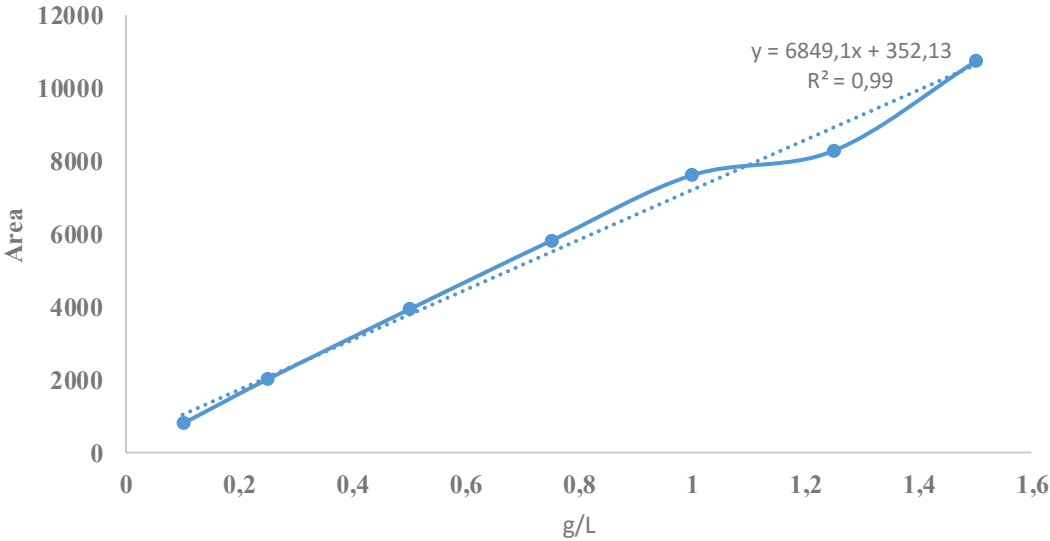
Annex 1. Potassium hydrogen phthalate standard curve for COD (KHP).



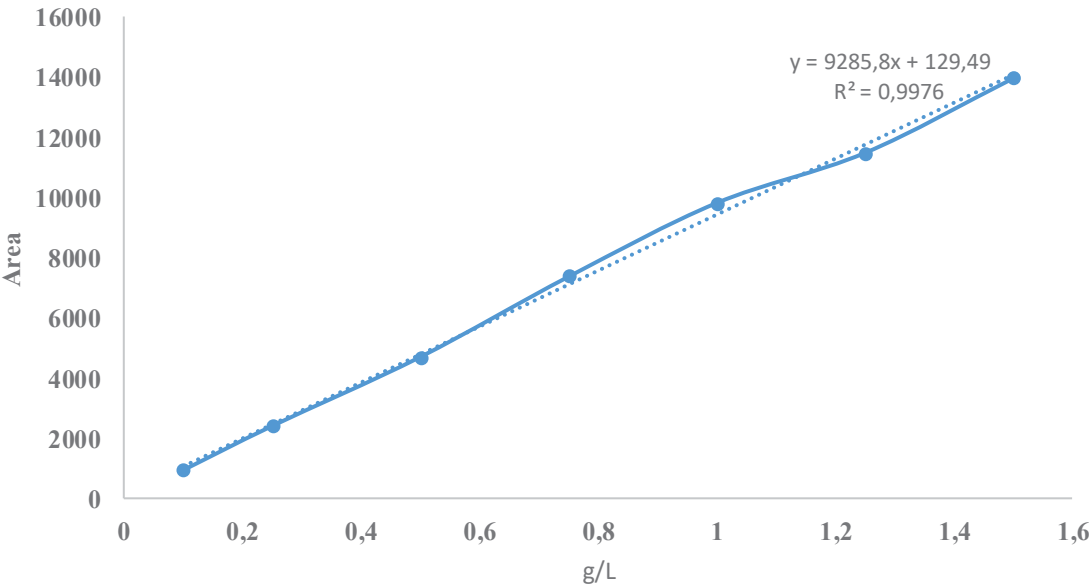
Annex 2. Standard maltose curve



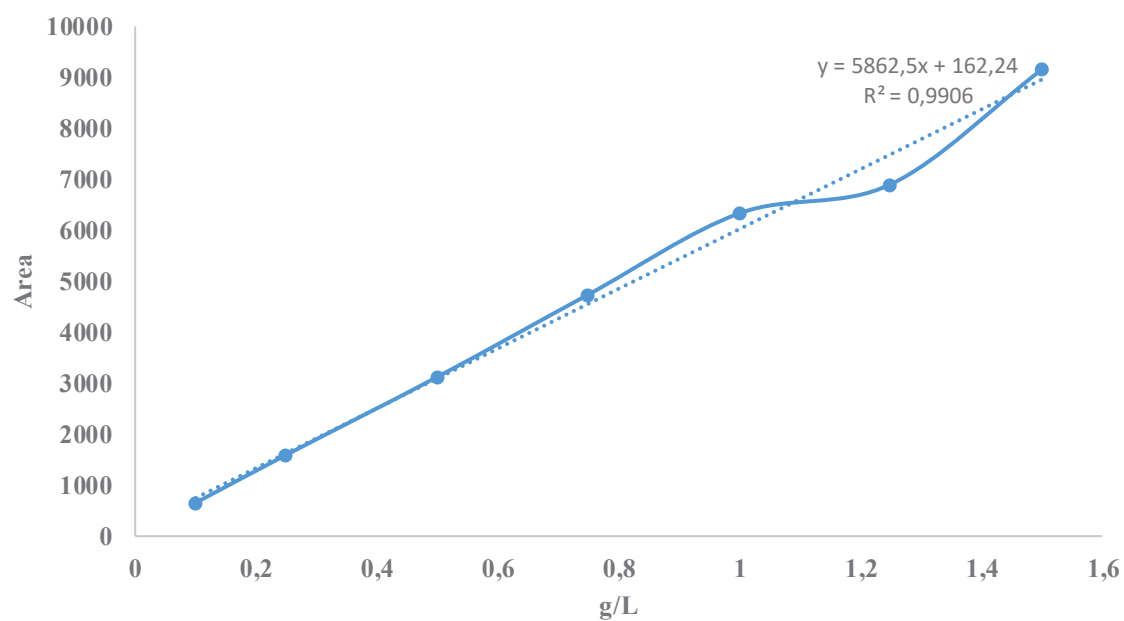
Annex 3. Standard glucose curve



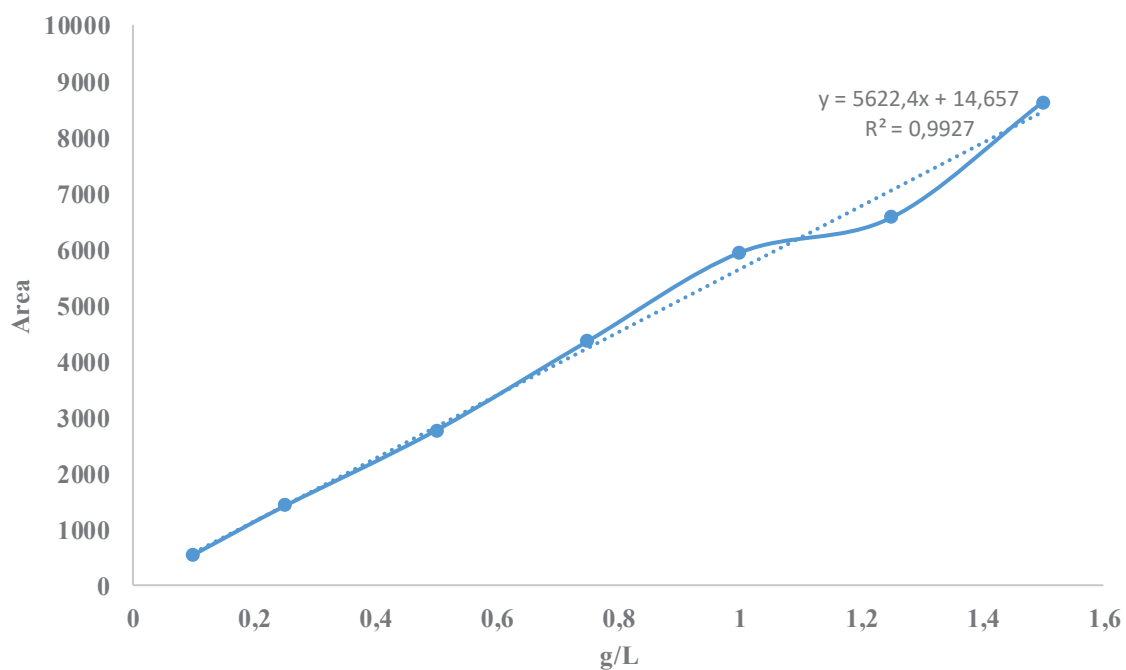
Annex 4. Standard fructose curve



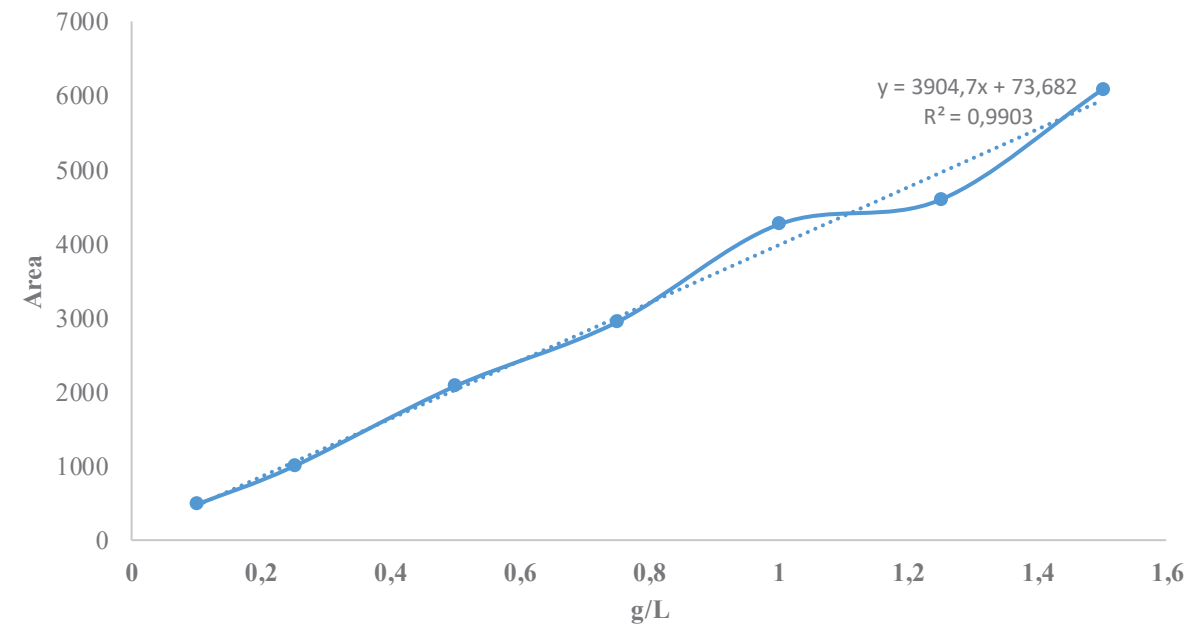
Annex 5. Standard succinic acid curve



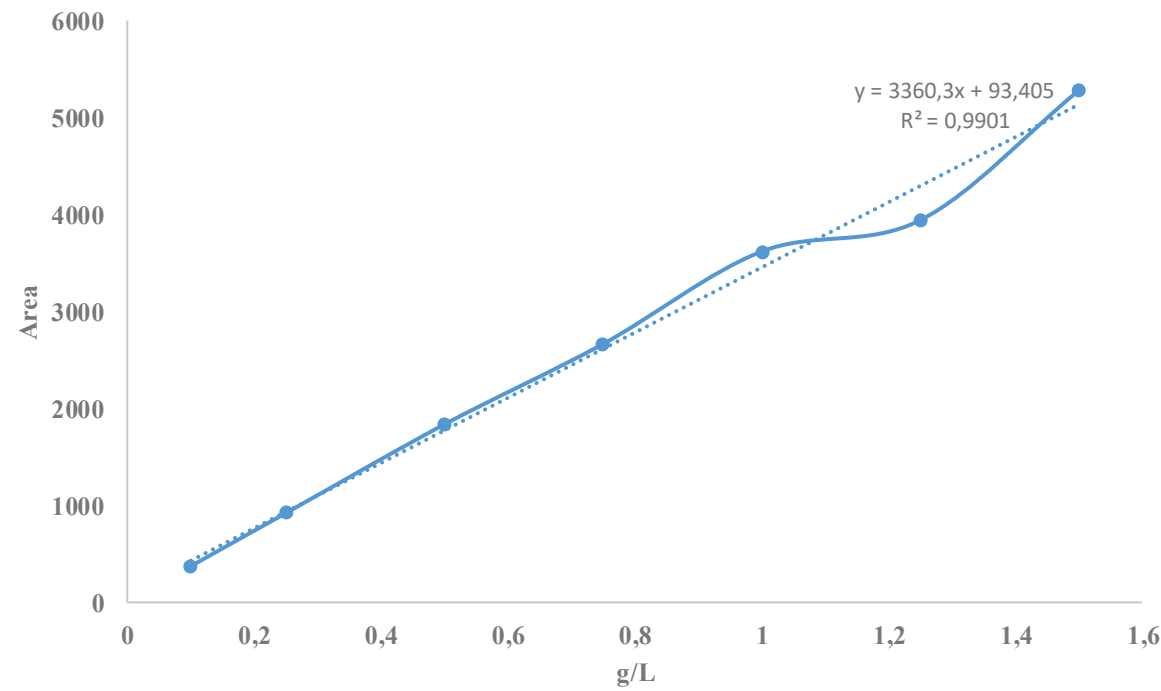
Annex 6. Standard lactic acid curve



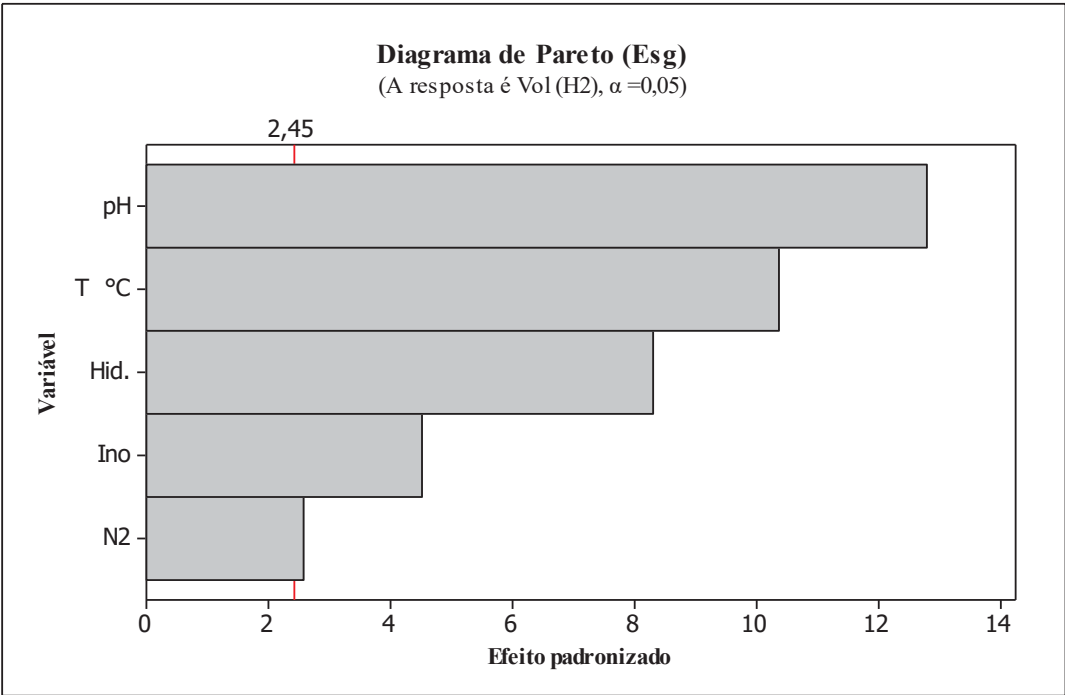
Annex 7. Standard acetic acid curve



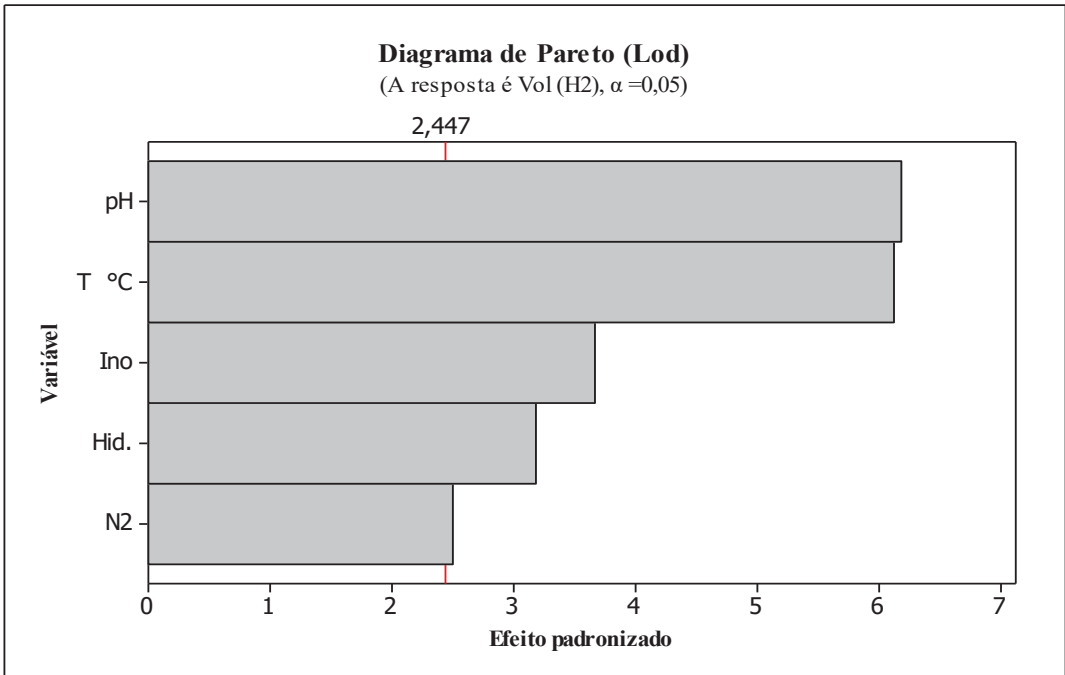
Annex 8. Standard ethanol curve



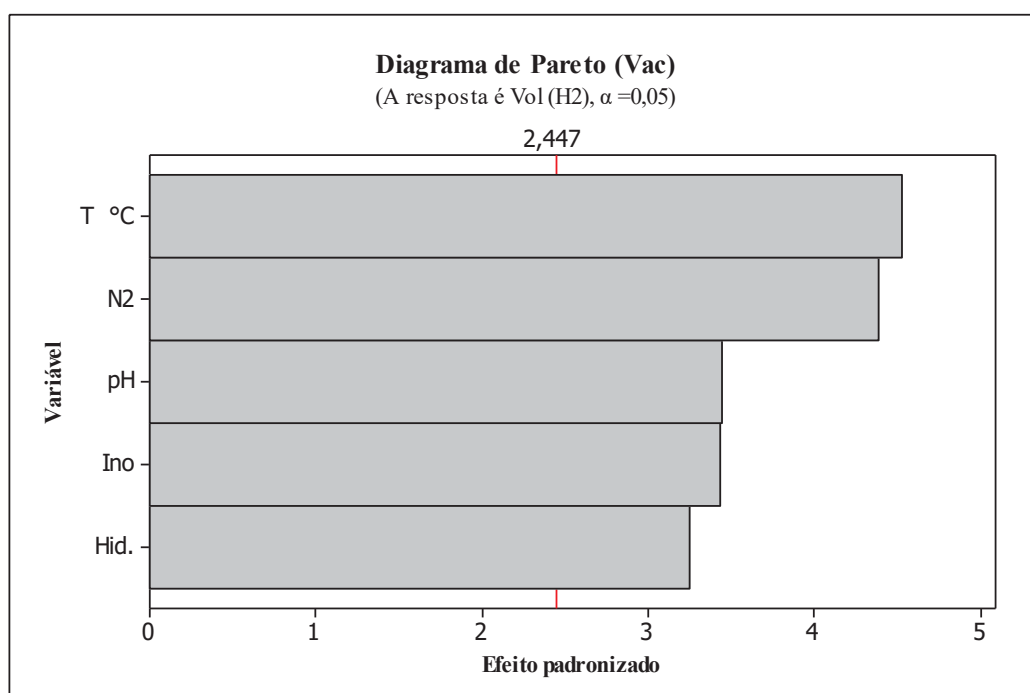
Annex 9. Pareto diagram for the *Esg* consortium



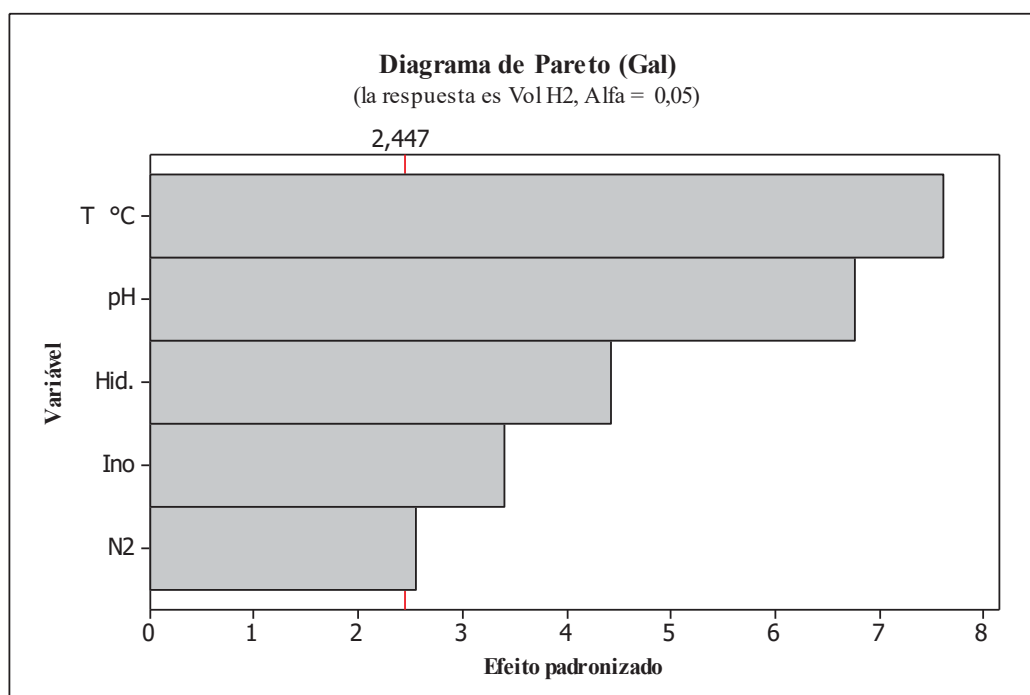
Annex 10. Pareto diagram for the *Lod* consortium



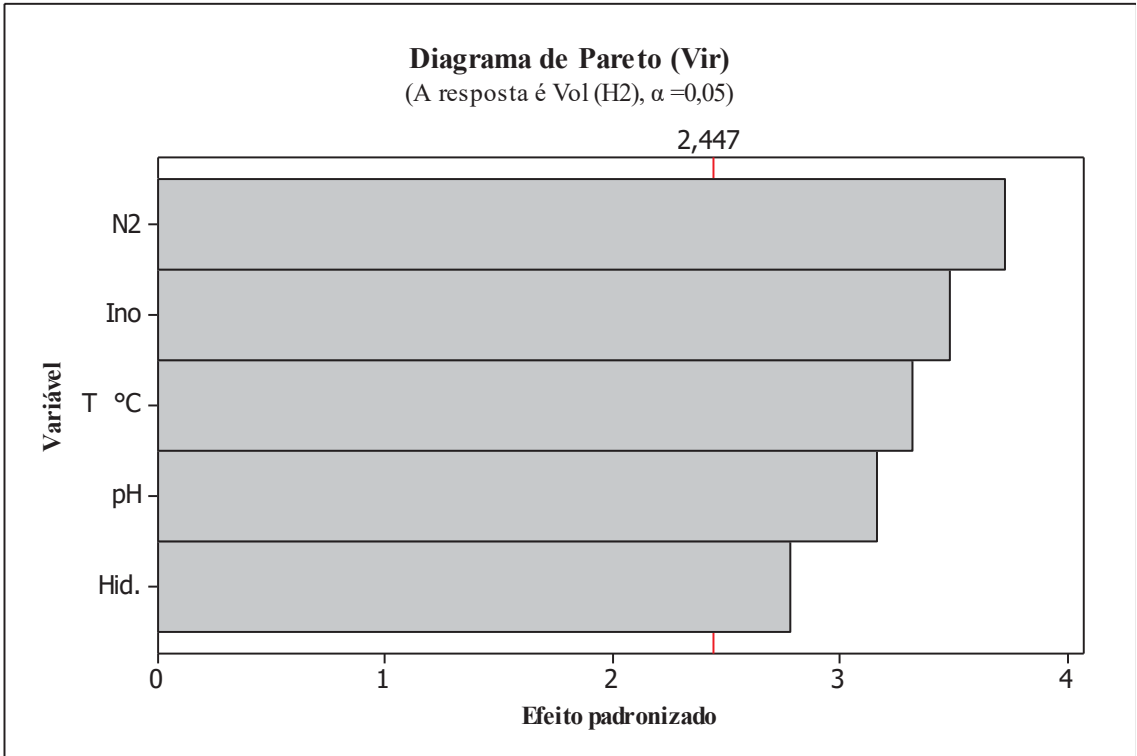
Annex 11. Pareto diagram for the *Vac* consortium



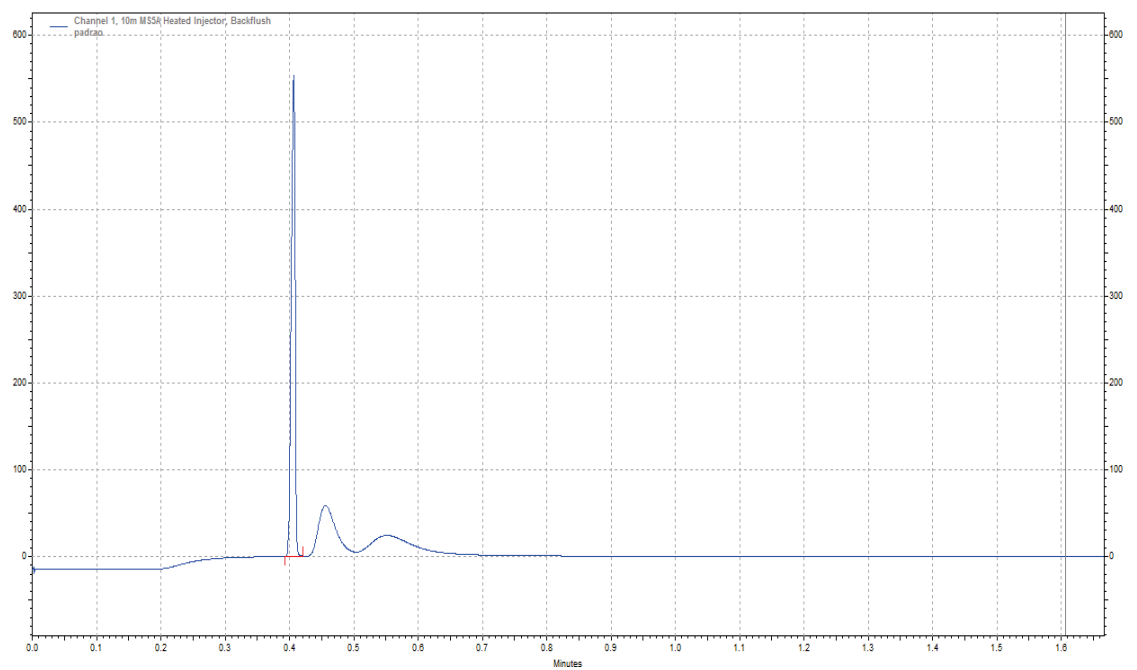
Annex 12. Pareto diagram for the *Gal* consortium



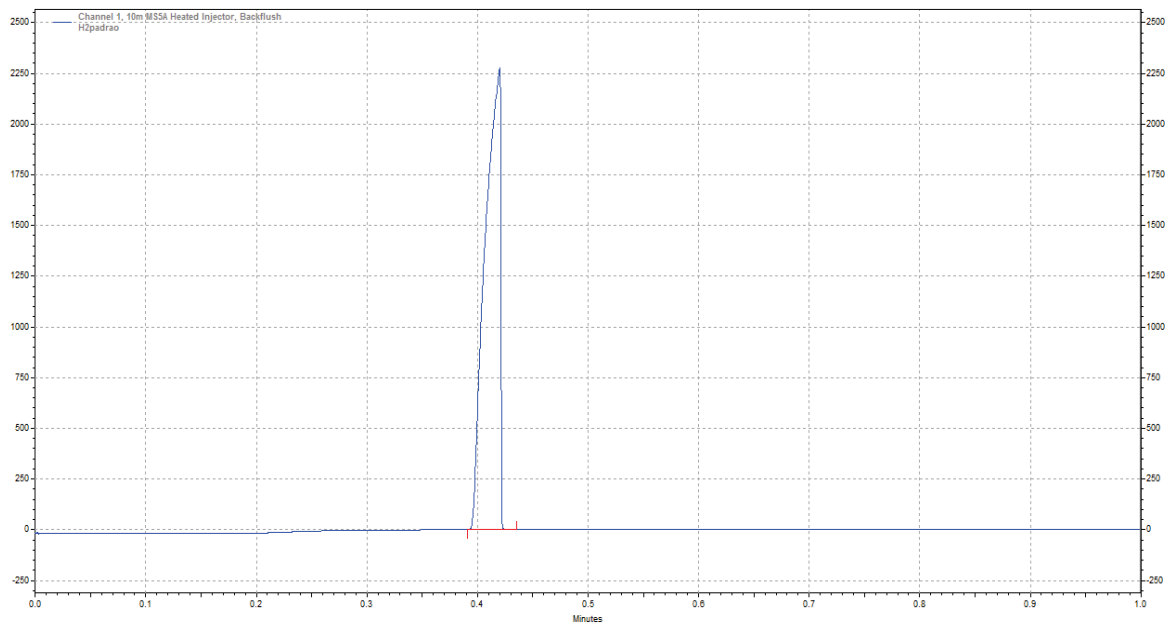
Annex 13. Pareto diagram for the *Vir* consortium



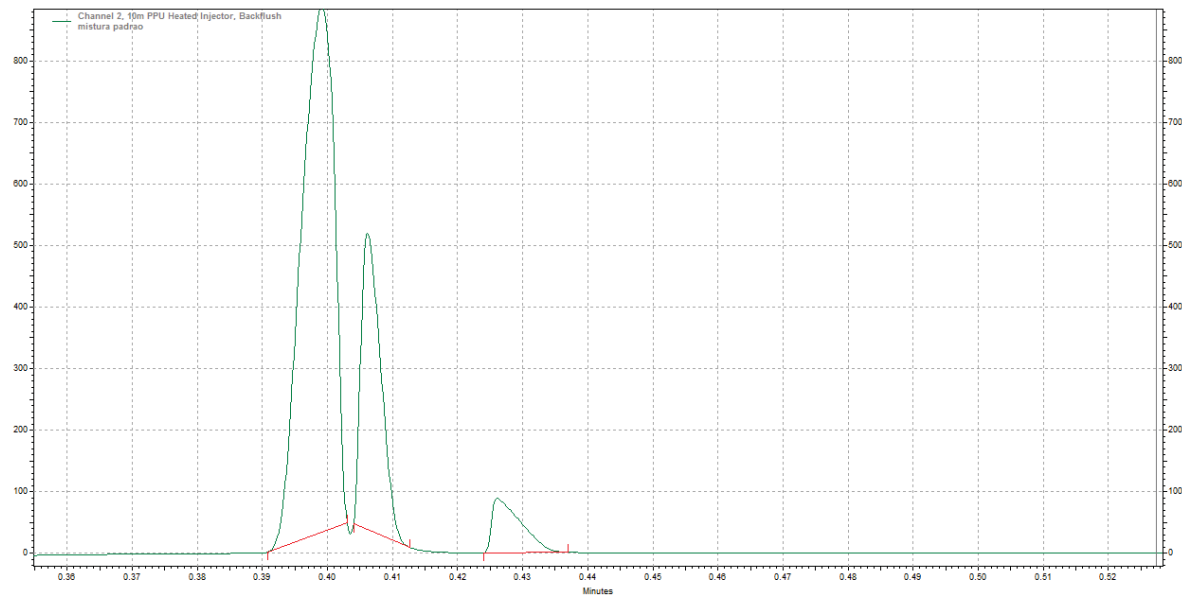
Annex 14. H₂ Standard 10%



Annex 15. H₂ Standard 99.999%



Annex 16. N₂, CO₂, CH₄ Standard



Annex 17. Quantification of hydrogen in a sample fermented by *Vir* consortium

