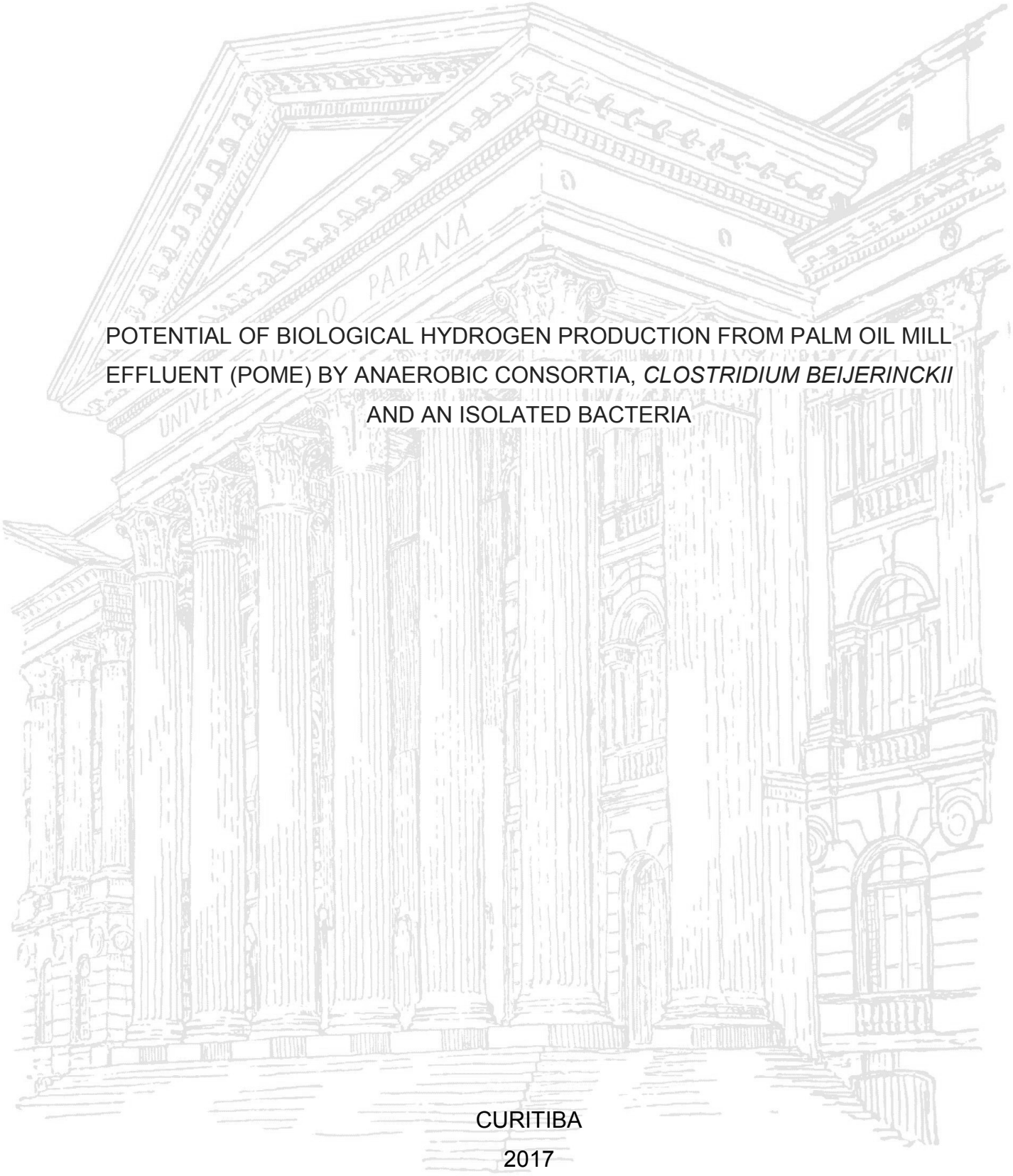
UNIVERSIDADE FEDERAL DO PARANÁ

DRIELLY ROSA



POTENTIAL OF BIOLOGICAL HYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) BY ANAEROBIC CONSORTIA, *CLOSTRIDIUM BEIJERINCKII* AND AN ISOLATED BACTERIA

CURITIBA

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Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, da Universidade Federal do Paraná.

Orientadora: Profª Dra. Adriane B. P. Medeiros

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **DRIELLY ROSA** intitulada: **Potential of Biological Hydrogen Production from Palm Oil Mill Effluent (POME) by Anaerobic Consortia, *Clostridium beijerinckii* and Isolated Bacteria**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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

Curitiba, 05 de Outubro de 2017.

ADRIANE BIANCHI PEDRONI MEDEIROS
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Os Avaliadores Externos Craig Faulds e Emmanuel Bertrand participaram por videoconferência - Ata homologada na 67ª Reunião de Colegiado realizada no dia 09 de novembro de 2017.

Universidade Federal do Paraná
Setor de Tecnologia
Divisão de Engenharia de Bioprocessos e Biotecnologia
Programa Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia

EXTRATO
Ata da 67ª Reunião do Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia

Aos nove dias do mês de Novembro do ano de dois mil e dezessete, reuniram-se os membros do Colegiado do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia (PPGEBB). Estavam presentes os professores: Júlio Cesar de Carvalho, Luciana Porto de Souza Vandenberghe, Adriane Bianchi Pedroni Medeiros, Adenise Lorenci Woiciechowski, Carlos Ricardo Soccol, Vanete Thomaz Soccol, Gilberto Vinicius de Melo Pereira, os professores convidados do DEBB Luiz Augusto Junior Letti e Cristine Rodrigues. Sob a presidência do professor Júlio César de Carvalho, coordenador do PPGEBB, que agradeceu a presença de todos, foi declarada aberta a sessão. Sob a presidência do professor Júlio Cesar de Carvalho, coordenador do PPGEBB, que agradeceu a presença de todos, foi declarada aberta a sessão. **2)Homologar Ata de Defesa de Mestrado (Biodev) dos alunos Drielly Rosa e Luiz Alberto Zevallos Torres corroborando a participação dos membros externos da França, que não assinam a Ata de Defesa: Professores doutores Craig Faulds, Emmanuel Bertrand,Laurence Lesage-Meessen, Jean Luc Cayol e Eric Record, (foi feito em videoconferência)** – O Colegiado aprovou por unanimidade.

Júlio C. de Carvalho
Prof. Dr. Júlio Cesar de Carvalho
Coordenador do PPGEBB

Marta H. D. Szadkoski
Marta Helena Szadkoski
Secretária Executiva do PPGEBB

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A Deus por me dar força e paciência para realizar esse trabalho; agradeço aos meus pais e meu namorado pelo suporte técnico, financeiro e emocional. Sem eles, nada disso seria possível.

À minha orientadora Adriane Medeiros por acreditar em mim e por seus conselhos. Esse mérito é nosso.

Ao departamento de Engenharia de Bioprocessos e Biotecnologia da UFPR, à CAPES e à empresa Vale pelo suporte e financiamento da minha pesquisa.

O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.

(José de Alencar)

RESUMO

O efluente da indústria de óleo de palma (POME) não fresco foi testado como um substrato para a produção de biohidrogênio em fermentação anaeróbia. Cinco consórcios microbianos diferentes, suas bactérias isoladas (as de alta concentração no consórcio e compatíveis com o meio seletivo) e uma cepa reconhecida pela produção de hidrogênio (*Clostridium beijerinckii*) foram inoculados em um meio a base de POME puro, diluído e hidrolisado, para comparar o rendimento da produção de hidrogênio. O planejamento experimental foi feito em tubos Hungate de 15mL, em uma proporção de 5mL de meio para um 1mL de inóculo. A produção de hidrogênio foi feita em uma escala maior dentro de um biorreator de 1L seguindo as mesmas proporções do meio e das condições de fermentação dos tubos. Quando a cepa ATCC 8260 (*Clostridium beijerinckii*) foi cultivada a 30ºC em POME hidrolisado P003, contendo 7,5g/L de sacarose, durante 8 dias de fermentação e com 20% de inóculo, o rendimento máximo da produção de hidrogênio foi 4,62 LH_2/L_{med}. Os melhores resultados foram com os experimentos em tubos devido ao pequeno volume do frasco e as melhores condições de controle.

Palavras-chave: POME. Fermentação anaeróbica. *Clostridium Beijerinckii*. Biohidrogênio. Tubos.

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ABSTRACT

Non-fresh Palm oil mill effluent (POME) was tested as a substrate to produce hydrogen in dark fermentation. Five different microbial consortia, and their isolated bacteria (the bacteria of higher concentration in the consortia and compatible with the selective medium), and *Clostridium beijerinckii* (ATCC 8260) a strain recognized as hydrogen producer were inoculated in a medium based in raw, diluted and hydrolyzed POME to compare the yield of biohydrogen production. The experimental planning was done in 15mL Hungate tubes in a proportion of 5mL of media to 1mL of inoculum. The hydrogen production was scale up to 1L bottle following the same proportion of medium and fermentation conditions. When the strain ATCC 8260 (*Clostridium beijerinckii*) was cultivated at 30ºC in the hydrolyzed POME (P003), containing 7.5g/L of sucrose, during 8 days of fermentation and 20% of the inoculum, the maximum biohydrogen production yield was 4.62 LH_2/L_{med} in tubes. The best results were with the experiments in the tubes due to the lower volume of the flask and better control condition.

Key-words: POME. Dark fermentation. *Clostridium Beijerinckii*. Biohydrogen. Tubes.

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SUMMARY

1 INTRODUCTION

The huge world energy demand is currently met basically by fossil fuels. This energy source has a negative environmental impact due to the emission of CO_2 and other gases, such as global warming and air pollution change (Azman *et al.,* 2016). Some alternatives are renewable and sustainable fuels, which can be clean and very interesting economically due the use of the cheapest feedstock and processes (Norfadilah *et al.*, 2016).

Hydrogen is an alternative for the fossil fuels demand because it has a high-energy content (120MJ/ Kg), being three times superior to hydrocarbon fuels (Barca *et al.*, 2016), and it can be obtained from industrial wastes (biological production) and its combustion results in water (Sá *et al.*, 2014). The highest hydrogen production is from fossil fuel and the process involves high electricity consumption, is expensive and not environmentally friendly. The biological production of hydrogen, an alternative process, can be done at room temperatures and pressures (Singh and Wahid, 2015), and it is an eco-friendly, low energy consuming approach compared to chemical processes.

Dark fermentation is a biological process in which biohydrogen is produced by microbial growth in carbohydrate-based substrates. Compared to other processes of hydrogen production, the bacterial anaerobic fermentation is the most attractive due the ability of strict or facultative anaerobes microorganisms to produce hydrogen and volatile fatty acids, such as acetate, butyrate, propionate, hydrogen sulphide and ethanol (Bedoya *et al.*, 2007; Krishnan *et al.*, 2016), from organic feedstocks (Barca *et al.*, 2016). One of the main problems observed in biohydrogen production by dark fermentation is the low substrate conversion efficiency and residual substrates present in acid-rich wastewater generated from the biohydrogen production process. The persistent accumulation of acidogenic by products such as VFA (volatile fatty acids) (Mamimin *et al.*, 2015) causes a decrease of the pH, resulting in process inhibition (Intanoo *et al.*, 2012).

Palm oil mill effluent (POME) is the liquid waste produced during the palm oil extraction process. POME has high organic matter content and is considered one of the most polluting wastewaters in the world, both in terms of composition and abundance. The residue is a viscous and brownish liquid, with large amounts of colloidal matter, is acidic (Ahmed *et al.*, 2015) and it has a high biochemical-oxygen-

demand (BOD) and chemical-oxygen-demand (COD). Estimations showed that more than 50 million m³ of POME is annually produced in the world (Krishnan *et al.*, 2016). The pH value of POME ranges from 3.7 to 4.5 and it is discharged at a temperature of 80-90ºC (Hossain *et al.*, 2016). The characteristics of POME changes per batches, days, climate and conditions of the process of palm oil (Ahmed *et al.*, 2015).

POME can be used as a renewable substrate for biological processes to produce biohydrogen. It can be obtained in abundance because the palm oil process generates tons of this wastewater (one ton of palm oil produces approximately 5.5 – 7.5 tons of POME) (Norfadilah *et al.*, 2016). The production of biohydrogen from POME is variable between reports, reaching yields as 5.988 LH_2/L_{med} (Norfadilah *et al.*, 2016), and 5.350 LH_2/L_{med} by Singh *et al.*, (2013) and still requires research due to its physicochemical characteristics and the high range of hydrogen-producing microorganisms in various agro-industrial substrates. Pure cultures, co-cultures and mixed consortia have been studied to improve biohydrogen production according to the carbon source (Mishra *et al.*, 2015).

POME has showed interesting characteristics in dark fermentation and hydrogen production. It is a rich sugar substrate, with lignocellulose structure, which can be broken by the acid hydrolysis and consumed by anaerobic bacteria (Azman *et al.*, 2016).

Several strains of bacteria have been found to convert carbon sources into biohydrogen in the dark fermentation process, such as *Escherichia, Clostridium, Bacillus* (Bedoya *et al.*, 2007) and *Enterobacter.* Strict anaerobic bacteria are the most popular microorganisms for biohydrogen production because of their ability to degrade a wide range of substrates in wastewaters and the higher capacity of biohydrogen production when compared with facultative microorganisms. Among these bacteria, the genus *Clostridium* is the major hydrogen producer (Azman *et al.*, 2016). It produces hydrogen mainly during the exponential growth phase (Tian *et al.*, 2016). During the stationary phase, the metabolism of this microorganism shifts from hydrogen/acid production to solvent production (Chong *et al.*, 2009).

Mixed cocultures or consortia are used mainly when complex material is used as a substrate to produce hydrogen (Nath and Das, 2011). This microbial groups have two characteristics: 1) the members of the consortia communicate with one another by exchanging metabolites and 2) promote the division of labor and degrading complex substrates (Xiao *et al.*, 2013). Economically, the use of consortia

is recommended because it does not require sterilized media and because it has less chances of contamination of the microbial culture (Nath and Das, 2011). Most mixed consortia contain species of *Clostridium* (Liu *et al.*, 2016).

This work aims the production of Biohydrogen from Palm Oil Mill Effluent (POME) by different anaerobic bacteria (consortia, strain and isolated form), comparing each other and evidencing the technical viability of this process, in order to propose an alternative source of energy and a future implementation at the Palm Oil Production Plant in Mojú / PA.

2 BIBLIOGRAPHIC REVIEW

Hydrogen is an effective alternative energy source that aims to reduce the fossil fuel dependency. This gas has a high specific energy when compared to others fuels and it is compatible with electrochemical and combustion process to conversion of energy (Dincer and Acar, 2015).

Besides these characteristics, the hydrogen is known to be the smallest element, to be very reactive and to be unstable as well as in normal temperature and pressure conditions. The hydrogen is inflammable, odorless, tasteless, colorless and diatomic. This gas has a high heat combustion and produces water when burned (Sreethawong *et al.*, 2010), which emphasizes its importance as a non-polluting fuel . The applicability of hydrogen varies from electricity generation and heat generation for use in internal combustion vehicle (Sá *et al.*, 2014).

Hydrogen is the most abundant element in the universe but it does not exist in its alone form in nature. The earth's surface contains approximately 0.14% hydrogen and the atmosphere contains 0.07% hydrogen (Das and Veziroglu 2001). Substances such as natural gas, water, hydrocarbons and biomass contain carbon-hydrogen or oxygen-hydrogen bonds, but it has low energy. The hydrogen-hydrogen bonds contain more energy and the methods to obtain this high-energy are complexes and demand high costs (Sydney, 2013).

2.1 SYSTEMS OF HYDROGEN PRODUCTION

The technologies to obtain hydrogen include a diverse set of primary energy sources, such as wind, solar, geothermal, nuclear and hydroelectric (Dincer and Acar, 2015), which can be used to extract hydrogen from water or others feedstock. Process such as stream reforming of hydrocarbons and electrolysis are chemical systems of hydrogen production and the main method to obtain hydrogen (Guo *et al.,* 2010).

The high-purity hydrogen can be obtained by other routes besides the stream reforming. The water-gas shift reaction is the most important industry process and especially used in ammonia synthesis (Ismail *et al.,* 2010). In the second plan are used the partial oxidation of coal, heavy residual oil and other refinery products of low-value such a hydrogen production capacity (Sydney, 2013).

2.2 ENVIRONMENTAL AND ECONOMIC SIGNIFICANCE

The increase of energy demands has resulted in sudden fossil fuel consumption. Hence, the level of pollution across the globe is increasingly and alarming. The greenhouse gases (GHGs) from the combustion of fossil fuels in turn aggravated the global warming. Combustion of fossil fuels emit about 6 Gigatons of carbon per year in the form of carbon dioxide to the atmosphere (Rasdi *et al.,*2012). Hydrogen is an important and promising energy source that can have a significant role in the reduction of greenhouse gas.

The hydrogen production was estimated to be $82.6 billion in 2010. Annually, the prospect is an increase of the volume production about 5,6% (between 2011 to 2016) due to rising demand of hydrogen-operated fuel cell applications (Rasdi *et al.,* 2012).

2.3 PALM OIL MILL EFFLUENT (POME)

Palm oil mill effluent (POME) is the liquid waste produced during the palm oil extraction process. Estimations showed that more than 50 million m^3 of POME is annually produced in the world. This is equivalent to a power capacity of 800GW (Krishnan *et al.,* 2016). The huge POME production impacts negatively the environmental due to its high organic matters and toxic characteristic (Azman *et al.,* 2016). The use of this product as a substrate for energy production would be an useful way to recover the present and future energy crisis (Hossain *et al.,* 2016).

Palm is a tropical plant inhabitant to Central and West Africa. Since the 14^{th} century, the palm oil has turned into a fundamental agricultural commodity in Indonesia and Malaysia, the first and the second largest producer of palm oil in the world respectively (Tabassum *et al.,* 2015). Studies showed that the production of one ton of crude palm oil requires 6-8 tons of water and over this, 50% ends up to wastewater (Norfadilah *et al.,* 2016).

Palm oil has many applications in several products, as soap, cooking oil, cosmetic and others. The high moisture content oil palm biomass is the advantage of thermal conversion process and can be a great source of hydrogen production (Hossain *et al.,* 2016).

The POME is produced and discharged from the three main stages of palm oil process: clarification (60%) sterilizer condensate (36%) and hydrocyclone wastewater (4%). Table 1 shows the characteristics of POME in the individual stages (Ahmed *et al.,* 2015).

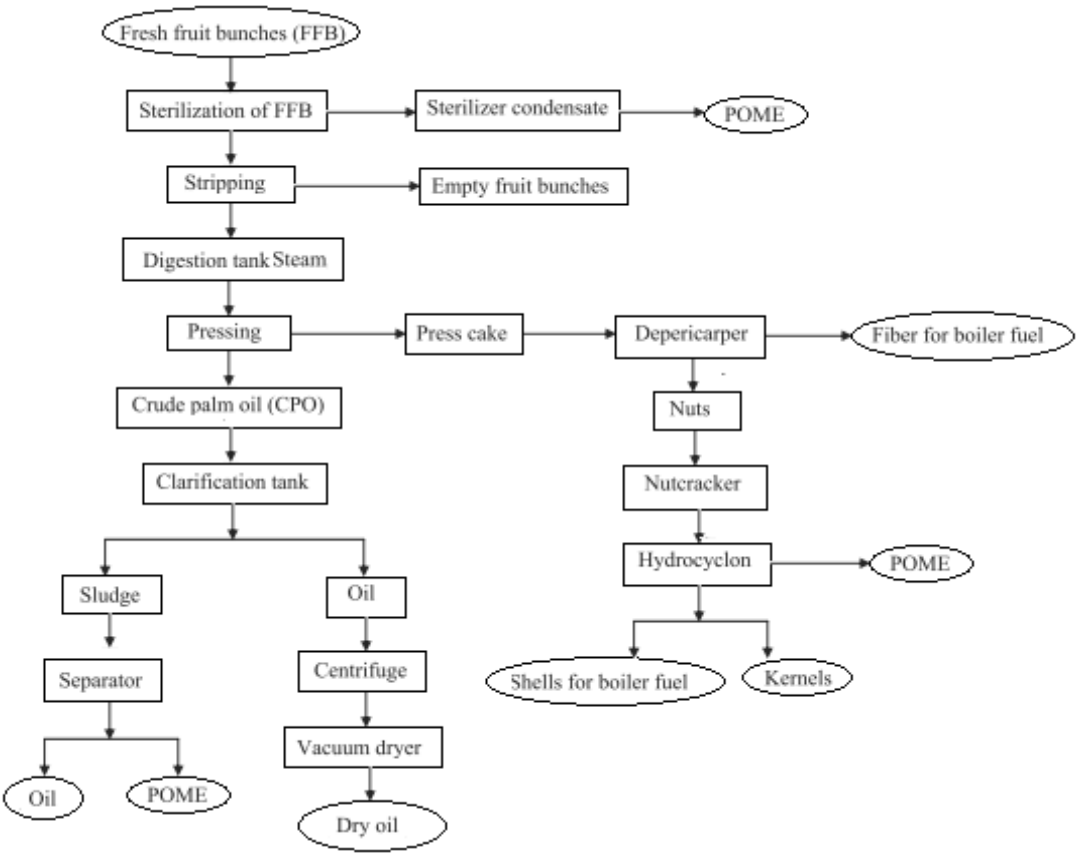
Table 1: Characteristics of individual wastewater streams in palm oil mill

Parameters	Sterilizer condensate	Clarification wastewater	Hydrocyclone wastewater
Chemical oxygen demand (COD) (mg/L)	47000	64000	15000
Biochemical oxygen demand (BOD_3, 30°C) (mg/L)	23000	29000	5000
Dissolved solids (DS) (mg/L)	34000	22000	100
Suspended solids (SS) (mg/L)	5000	23000	7000
Total nitrogen (TN) (mg/L)	500	1200	100
Ammoniacal nitrogen (mg/L)	20	40	-
Oil and grease (mg/L)	4000	7000	300
pH	5.0	4.5	-

Source: Adapted from Ahmed *et al.,* (2015).

The clarification step is the responsible for the highest POME obtainment, and this demands a huge volume of water. Hence, a huge volume of the palm oil mill effluent is produced (Wongfaed *et al.,*2015). Fig 1. shows a chart of the common extraction process of palm oil and POME generation.

Figure 1: Flow diagram in a typical palm oil process



Source: Adapted from Ahmed *et al.,* (2015)

Among the steps of palm oil process are the reception, transfer and storing of fresh fruit bunches (FFB), which is the main feedstock of this production. The sterilization of FFB occurs at 140ºC for 75-90 min and at a pressure of 293.84kPa; the stripping, digestion and extraction of crude palm oil (CPO); the clarification and purification of the crude palm oil to separate the fibrous materials and the oil and finally, the separation of kernels and drying (Ahmed *et al.,* 2015).

2.3.1 POME characteristics

The palm oil process generates a high polluting wastewater, known as palm oil mill effluent (POME). In the nature, POME is viscous, trick brownish, voluminous colloidal matter, acidic (Ahmed *et al.,* 2015) and it has a high biochemical-oxygen-demand (BOD) and chemical-oxygen-demand (COD). The pH value of POME is 3.7 e 4.5 and it is discharged at a temperature of 80-90ºC (Hossain, Jewaratnam, and Ganesan 2016). The characteristics of POME are described in Table 2.

Table 2: Characteristics of raw POME

Parameters	Reference						
	Ahmed et al (2014)	Bhatia et al (2007)	Ismail et al (2010)	May et al (2013)	Norfadilah et al (2016)	Rupani et al (2010)	Singh et al (2013)
Biological oxygen demand - BOD_5 (mg/l)	42000	-	-	45357	37750	25000	39150
Chemical oxygen demand - COD (mg/l)	19000	40200	94400	73498	69500	50000	70700
Total solids (mg/l)	11666	39470	-	56279	47690	40500	36000
Suspended solids (mg/l)	-	17927	17800	32005	30870	18000	-
Dissolved solid (mg/l)	18670	-	-	-	-	-	-
pH	4.5	4.5	4.5	4.5	3.4	4.7	4.5
Temperature (°C)	85	80	-	-	80	85	-
Total nitrogen (mg/l)	600	800	800	760	692	750	865
Ammoniacal nitrogen (mg/l)	20	-	-	69	-	35	30
Oil and grease (mg/l)	3766.67	2658	10100	6670.5	8370	4000	2250

Source: The author (2015).

The organic matter of POME is very high, varying from 19,000 to almost 95,000 mg/L (COD) of each effluent sample. This variation occurs due to the process of palm oil, which is distinct among producing regions, as well as the parameters used in production (Azman *et al.,* 2016).

POME is a mixture of carbohydrates and it has been found that the raw substrate contains 38.36% cellulose, 23.21% hemicellulose and 26.72% lignin. This represents a low-cost of sugar source (Ali Amat *et al.,* 2015). Therefore, an efficient alternative to release the fermentative sugars is by the acid hydrolysis method (Azman *et al.,* 2016).

2.4 WORLD AND BRAZILIAN ASPECTS OF PALM OIL AND POME PRODUCTION

The consumption of palm oil in the world has improved and is controlled basically by Indonesia and Malaysia. In 2014 the production of palm oil in the world

was 62,34 million tons and 85% of this production comes from these two countries. By 2020, it is expected to increase to 78 million tons and in 2043 with the population growth, the demand of palm is estimated in 360 million tons. This demand will produce a huge volume of POME, which could be utilized to energy production. Studies calculated that around 28m³ of biogas, such as biohydrogen and biomethane is generated from 1m³ of POME (Ahmed *et al.,* 2015).

The characteristics of POME changes per batches, days, climate and conditions of the process of palm oil. In Malaysia, the Environmental Department purposed a regulatory control over discharges from palm oil mills since 1984 (Ahmed *et al.,* 2015; Ali Amat *et al.,* 2015). Equally, the Brazilian Ministry of Environment also has discharge standards of effluent into water sources. The *Conselho Nacional do Meio Ambiente (CONAMA)* (Brasil, 2005; Brasil, 2011), ministry organization in Brazil created two laws on this subject. The compassion between Malaysia and Brazil standards are in Table 3.

Table 3: Discharged standards of POME into water source in Malaysia and Brazil

Parameters	**Limits of discharge according to standards**	
	Malaysia - 1/1/1984	Brazil - CONAMA Nº 357/2005 and Nº 430/2011
Biochemical oxygen demand (BOD_3 30°C) (mg/L)	100	-
Biochemical oxygen demand (BOD_5 20°C) (mg/L)	-	Remove 60% of initial value
Total dissolved solids (mg/L)	-	500
Suspended solids (mg/L)	400	-
Total nitrogen (mg/L)	200	-
Ammoniacal nitrogen (mg/L)	150	3,7 to pH ≤ 7.5
Oil and grease (mg/L)	50	50
pH	5 to 9	5 to 9
Temperature (°C)	45	<40

Some parameters have the same value for each country and this was establishing due to the high potential of pollution of the wastewater like POME in water body. In Brazil there is no specific law with standards to POME discharge. The values of the table refers to general effluents (Brasil 2011; Brasil 2005).

2.5 BIOHYDROGEN PRODUCTION

The biological hydrogen production can be done through direct biophotolysis of water, indirect biophotolysis of water by cyanobacteria, photofermentation, dark fermentation (or anaerobic fermentation) and hybrid systems utilizing photosynthetic and anaerobic bacteria (Das and Veziroglu 2008).

The direct biophotolysis of water is made by green algae in anaerobic condition with the presence of light, aiming the water decomposition and hydrogen production. The indirect form involves the cyanobacteria which uses the carbon energy of photosynthesis to generate hydrogen from water. The photofermentation is done by no-sulfur bacteria which utilizes light energy to transformer organic acid in hydrogen and carbon dioxide (Das and Veziroglu 2008).

Dark fermentation is a biological process in which biohydrogen is produced by microbial growth in carbohydrate-based substrates. This process demands a anaerobic condition under the lack of light and can be operated in mesophilic, thermophilic and hyperthermophilic conditions, depending on the microorganism used (Wang and Wan 2009). Compared to other process, the bacterial anaerobic fermentation is the most attractive due the ability of strict or facultative anaerobes microorganisms to produce hydrogen and volatile fatty acids, such as acetate, butyrate, propionate, hydrogen sulphide and ethanol (Krishnan *et al.,* 2016; Bedoya *et al.,* 2007), from organic feedstocks (Barca *et al.,* 2016). Methane production is the second-stage (Krishnan *et al.,* 2016). Table 4 shows the biological process of hydrogen production (Sá *et al.,* 2014).

Table 4: Advantages, disadvantages and main microorganisms used for hydrogen production

Biological process	Advantages	Disadvantages	Microorganisms
Direct biophotolysis	Hydrogen production from water	Requires contant light	*Chalamydomonas reinhardii*
	Do not need ATP	Hydrogenases inhibition by oxygen	*Platymonas subcordiformis*
Indirect biophotolysis	Hydrogen production from water		Plectonema boryanum
		Requires contant light	*Anabaena siamensis*
		Needs ATP for the nitrogenases	*Anabaena variabilis*
	Ability to nitrogen fix and hydrogen production by nitrogenase	Presence of CO_2 in the gas	*Synechocysis sp.*
			Cyanothece sp.
			Nostoc sp.
Photofermentation	Utilized several wastes such substrate		*Rhodopseudomonas palustres*
		Requires light	*Rhodobacter sp.*
	Utilized huge light espectrum by photosynthetics bacteria	Presence of CO_2 in the gas	*Rhodobacter sphaeroides*
			Rhodobacter capsulatus
Anaerobic fermentation	Utilized several carbon source such substrate		*Clostridium sp.*
		Effluent treatment after fermentation	*Clostridium butyricum*
			Clostridium beijerinckii
	Do not need light		*Citrobacter freundii*
			Enterobacter cloacae
	Intermediate production of value-added metabolites	Presence of CO_2 in the gas	*Enterobacter aerogenes*
			Escherichia coli
			Klebsiella pneumoniae

Source: Adapted from Sá *et al.,* (2014).

Basically, the hydrogen production by anaerobic bacteria depends of the substrate, microorganism and process conditions such as pH, temperature, hydraulic retention time (HRT) and partial pressure of the gas (Bedoya *et al.,* 2007). The hydraulic retention time (HRT) is the most important factor to control variables influencing hydrogen production. A longer fermentation period induces a metabolic shift from acidogenesis to methanogenesis, which is considered unfavorable for biohydrogen production. Maintaining a shorter HRT helps restrict methanogenic bacteria growth as well as activity (Jung *et al.,* 2011).

The partial pressure of hydrogen influences the hydrogenase enzyme activity due to the end product inhibition. The hydrogen production is limited by the thermodynamics of hydrogenase reaction (Jung *et al.,* 2011).

2.6 METABOLIC PATHWAY OF MICROORGANISMS IN ANAEROBIC FERMENTATION

The glycolysis is considered the primary metabolic pathway where a substrate is converted to pyruvate, a central molecule of microbial fermentation. During anaerobic fermentation, pyruvate has a diverse fate under based operating conditions. Pyruvate enters the acidogenic pathway and generates volatile fatty acids (VFA) in association with the hydrogen production, according to Eq. [1], [2], [3] and [4] (Chen *et al.,* 2006).

$C_6H_{12}O_6 + 2H_20 \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (acetic acid) [1]

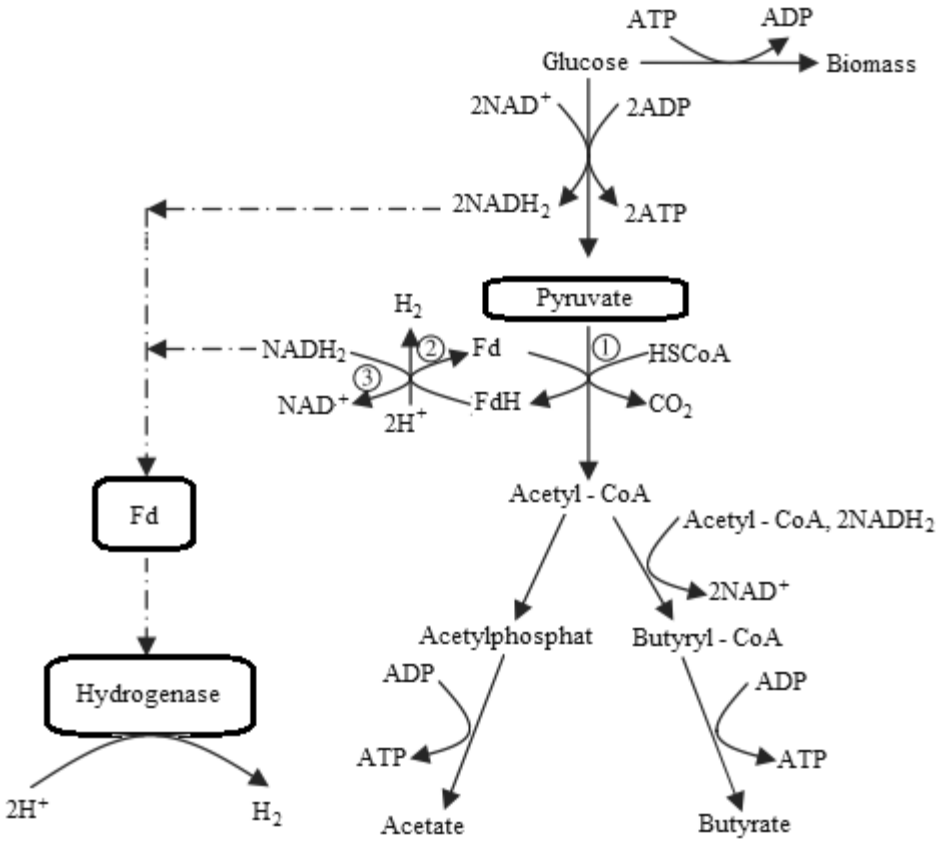
$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (butyric acid) [2]

$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$ (propionic acid) [3]

$C_6H_{12}O_6 \rightarrow CH_3CH_2OH + CO_2$ (ethanol) [4]

Dark fermentative biohydrogen production is considered the most practical among the various methods. It utilizes organic substrates as carbon source of energy and electrons. Biochemical reactions are currently known to generate biohydrogen in dark fermentation. The metabolic pathway of the *Clostridium butyricum* is shown in Fig 2 (Chen *et al.,* 2006).

Figure 2: Metabolic pathway of glucose by *Clostridium butyricum* under anaerobic conditions. 1) Pyruvate: ferredoxin oxidoreductase (PFOR); 2) Hydrogenase; 3 NADH: ferredoxin oxidoreductase



Source: Adapted from Chen *et al.,* (2006).

Hydrogenase and nitrogenase are the two most important enzymes involved in hydrogen production by fermentative process and are responsible for the reduction of monoatomic hydrogen to diatomic hydrogen. The hydrogenase is responsible for producing the hydrogen and can be classified in three groups: Ni-Fe- hydrogenase, hydrogenase metal free and Fe-hydrogenase, which has the role to remove the excessive equivalents (H^+) in strict anaerobes and could be inhibited for the oxygen presence (Bedoya *et al.,* 2007).

According to the stoichiometry of glucose oxidation, 12 mol of biohydrogen can be generated from 1 mol of glucose. The maximum yield in dark fermentation is 4 mol H_2/mol glucose, that is, only 33% of the stoichiometric maximum. The low biohydrogen yield is linked to microbial metabolism. However, this route is significantly affected by several factors, such as H^+, concentration, NADH/NAD^+ ratio, hydrogen partial pressure and temperature (Ren *et al.,* 2006).

2.7 PROCESS LIMITATION

One of the main problems observed in biohydrogen production by dark fermentation is the low substrate conversion efficiency and residual substrates present in acid-rich wastewater generated from the biohydrogen production process. Approximately 60–70% residual organic carbon remains in the effluent after dark fermentation and it requires further treatment prior to discharge. The persistent accumulation of acidogenic by products such as VFA (volatile fatty acids) (Mamimin *et al.,* 2015) causes a decrease of the pH, resulting in process inhibition. Biological limitations such as biohydrogen end product inhibition, acid or solvent accumulation, and hydrogen partial pressure limit the process efficiency (Intanoo *et al.,* 2012).

Hydrogen partial pressure can have a major impact on biological process performance because it indirectly plays a critical role in the biochemical equilibrium of the substrate conversion to biohydrogen and consequently in determining the metabolic pathway. When the dissolved hydrogen reaches a critical concentration (or partial pressure of 60kPa), the bacterial metabolism shift and the production of hydrogen, for example, can be decreased. Gas sparging can be an efficient technique to maintain maximum hydrogen production even though it leads to biogas dilution and higher cost for hydrogen recovery (Beckers *et al.,* 2015).

2.8 BIOHYDROGEN PRODUCTION BY WASTEWATER AND RENEWABLE SOURCES

A variety of sugar and carbohydrate sources including sucrose, glucose, xylose, molasses and others have been used for biohydrogen production by anaerobic fermentation (Ren *et al.,* 2006). Beside these, wastewater of various sources can be used in this process. The food-processing, dairy-based and alcohol-based industries are responsible for producing wastes of several nature, such as prepare the feedstock, equipment industrial plant, etc (Lin *et al.,* 2012). About this wastewater, the lignocellulose, a fibrous structure, represents the most renewable sugar source and is mainly composed of cellulose, hemicellulose and lignin (Ho *et al.,* 2012). These polymeric structures are found in wastewater of agroindustry process, coming from the plants. However, the lignocellulose should be

depolymerized to release the soluble sugars which are utilized as an energy source in dark fermentation (Azman *et al.,* 2016) .

2.9 ANAEROBIC MICROORGANISMS BIOHYDROGEN PRODUCER

Several strains of bacteria have been found to convert carbon sources into biohydrogen in the dark fermentation process, such as *Escherichia, Clostridia, Bacillus* (Bedoya *et al.,* 2007) and *Enterobacter.* Among these bacteria, the genus *Clostridium* is the major hydrogen producing microorganism in anaerobic fermentation (Azman *et al.,* 2016). The dark fermentation is divided into four stages: hydrolysis, acidogenesis, acetogenesis and eventually methanogenesis. Biohydrogen is produced during the acidogenesis and acetogenic phase (Hiligsmann *et al.,* 2011).

During the hydrolysis extracellular enzymes are produced, and it degrades complex particulate matter to simple matter. Acidogenesis occurs when the simple matter is metabolized in the bacteria cell and it is converted to organic acids, alcohol, carbon dioxide, hydrogen and new bacterial cells. In this phase, the main product is generated and the process needs to be limited. In the next step, acetogenesis, some products of the previous phase are converted in acetic acid (including hydrogen) and the methanogenesis converts all products in methane (Shi, Li, and Yu 2015).

One alternative to check the hydrogen production during the fermentation process is to follow the acetic acid formation, because the more the acid is produced, more hydrogen is being consumed. A method to limit the biohydrogen consumption in the fermentation, mainly with an anaerobic consortia strain, could be increasing the temperature or decreasing the pH of this process, because methanogenics bacteria does not produce spores in anaerobic condition, and die in extreme conditions (Sá *et al.,* 2014).

The hydrogen-producing microorganisms can be classified based on their oxygen sensitivity and temperature. Microorganisms that strictly require anaerobic conditions are called obligate anaerobes (Chong *et al.,* 2009). Microorganisms that can sustain anaerobic and aerobic environments are called facultative anaerobes. Facultative bacteria are always more advantageous to perform experimental work on obligate anaerobes, since they are easier to cultivate and can be kept in a laboratory (Hassan and Morsy 2015). Furthermore, based on their temperature requirements,

they can be further classified as mesophiles, which require room temperature for growth, or thermophiles that are adapted to higher temperatures (Das and Veziroglu 2008). In nature, hydrogen can be produced by pure microbial species or by consortia. Some members of the community can produce hydrogen while others can efficiently consume hydrogen for energy purposes, which in terms of hydrogen gas production is undesirable (Chong *et al.,* 2009).

2.9.1 Facultative anaerobic bacteria

Microorganisms used to produce hydrogen, such as anaerobic bacteria, are responsible for the degradation of organic material and require time to adapt to the new environment before beginning the substrate consumption and growth. The efficiency of the system depends on the microbial community, the substrate and environmental factors, pH and temperature (Ahmed *et al.*, 2015).

In the presence of oxygen, facultative anaerobes can produce ATP by aerobic respiration. In the absence of oxygen, they can produce ATP by anaerobic fermentation. Bacteria of the genus *Enterobacter sp*. are facultative anaerobes that can produce hydrogen under anaerobic conditions (Hassan and Morsy, 2015). These microorganisms have several properties that favor the production of biohydrogen. Facultative Anaerobes are known to produce a higher yield of hydrogen (Patel *et al.*, 2012).

2.9.2 Obligate anaerobic bacteria

Restricted anaerobic bacteria are the most investigated and used for biohydrogen production because of their ability to degrade a wide range of carbohydrates, which includes wastewater. In addition, compared to facultative anaerobes, they also produce a higher rate of hydrogen production. *Clostridia* species have been widely used to produce gas. It produces hydrogen mainly during the exponential growth phase (An *et al.*, 2014). During the stationary phase, the metabolism of this microorganism shifts from hydrogen to acid production. Among these bacteria, *Clostridia saccharoperbutylacetonicum, C. tyrobutyricum, C. butyricum, C. acetobutyricum, C. beijerinckii, C. thermolacticum, C. thermocellum* and *C. paraputrificum* are examples of spore-forming hydrogen producers under anaerobic conditions (Tian *et al.*, 2016).

2.9.3 Thermophiles

Thermophiles are obligate anaerobes found in various geothermal heated regions of earth, such as hot springs and deep-sea hydrothermal vents. Their culture requirements differ depending on the isolation source. Further, because they are obligate anaerobes, reducing agents such as L-cystine HCl are required in the media to remove even trace quantities of oxygen from the medium (Sydney 2013). Thermophiles can utilize a broad range of substrates such as cellulose and hemicelluloses. Typical examples of this group include genera *Thermoanaerobacter*, and *Thermotoga.* All members of this genus are able to utilize complex carbohydrate and proteins for growth and fermentative hydrogen production (Pandey *et al.,* 2013).

2.9.4 Anaerobic consortia

Mixed cocultures or consortia are used mainly when complex material is used as a substrate to produce hydrogen (Nath and Das 2011). Consortium use provides two essential functions. First, members of the consortium communicate with one another by exchanging metabolites or exchanging dedicated molecular signals, which allows the second important feature - division of labor, degrading the numerous complex substances (Xiao *et al.,* 2013). Cultures mixed to produce hydrogen from organic waste may be more advantageous because pure cultures can easily contaminate with hydrogen-consuming bacteria. In fact, for economic reasons, the industrial production of hydrogen using readily available complex raw materials is usually carried out under non-sterile conditions. Mixed microbial consortia can solve this problem once they have been selected for growth and dominance in non-sterile conditions (Nath and Das 2011). They are potentially more robust to changes in environmental conditions such as pH and temperature. Cultures blended as an inoculum to produce hydrogen can be isolated from a variety of sources, such as fermented soybean meal or sludge from anaerobic digesters from municipal sewage or organic waste and cooking waste sludge. Most mixed consortia contain species of *Clostridium* (Liu *et al.,* 2016).

2.10 BIOHYDROGEN PRODUCTION USING POME

The POME has showed interesting characteristics to dark fermentation and hydrogen production. It is a rich sugar substrate, with lignocellulose structure, which can be broken and consumed by anaerobic bacteria (Lee *et al.,* 2015). But different hydrogen yields are obtained according to the combination of substrate, microorganism and process conditions. Different equipment has been used to improve the process (Poh *et al.,* 2014).

The most recommended anaerobic digestion of POME includes anaerobic filters and anaerobic fluidized bed reactors, up-flow anaerobic sludge blanket reactors (UASB), expanded granular sludge blanket (EGSB), anaerobic baffled reactors (ABR), anaerobic sequencing batch reactor (ASBR), continuous stirred tank reactor (CSTR) and up-flow anaerobic sludge-fixed film reactor (UASFF). Because of the anaerobic conditions, one of the products of this process is the biohydrogen. But anaerobic digestion or POME treatment also can be done (Ahmed *et al.,* 2015). Biofilm reactors have been tested for hydrogen production from synthetic and real wastewater, showing several advantages compared to systems of biomass suspension. The biofilm provides a good protection of bacteria cells against sudden change of temperature, pH, organic load, etc (Barca *et al.,* 2016).

2.10.1 Aspects of POME treatment

The use of bacteria for the POME treatment in dark fermentation processes is common. However, some fungi may help reduce the organic matter of this wastewater. The fungus *Trichoderna viride* is reported to reduce over 91% of POME BOD_5, but there are no reports of biogas production with this method (Hallenbeck 2009). Another technique to POME treatment aims to remove the organic matter and color using membranes. Nanofiltration, for example removes molecules with weight of 200-1000g/mol, such as lignin contained in the POME (Ali Amat *et al.,* 2015).

2.10.2 Reports of hydrogen production from POME

Two-stages (thermophilic and mesophilic) continuous with recirculation of the digestive sludge in the dark fermentation process from POME is an alternative to produce hydrogen and methane (Ng, Lim, and Chan 2016). The POME is obtained in

the industry at a high temperature around 80-90ºC in raw form. Therefore, the thermophilic condition in a dark fermentation eliminates the need for cooling systems, which favors thermodynamics systems and maintains low hydrogen pressure. The result of biohydrogen production method is a high hydrogen and methane yields (Krishnan *et al.,* 2016).

Some recent studies about the use of POME to hydrogen production have been reported (Table 5). Utilizing different methodology, the researchers are reaching interesting results.

Table 5: Reports of hydrogen production from POME

METHOD	DESCRIPTION	HYDROGEN PRODUCTION AND CONDITIONS	REFERENCE
Production of hydrogen from dilute acid-hydrolyzed palm oil mill effluent in dark fermentation	. Empirical model . Chloride acid 37% . *Clostridium acetobutylicum* strain	108.35 ml H_2/g total reducing sugars consumed 333.5 ml cumulative hydrogen	Azman *et al.*, 2016
Production of hydrogen and methane from palm oil mill effluent using thermophilic and mesophilic fermentation	. Reactor UASB (thermophilic stage) - 2 days . Reactor CSTR (mesophilic stage) - 5 days . *Thermoanaerobacterium* species and *Methanobrevibacter sp*.	1.92 L H_2/L.d 3.2 L CH_4 /L.d	Krishnan *et al.*,2016
Biohydrogen production from palm oil mill effluent	. Microflora (seed sludge) . Bioreactor under mesophilic operation . Different ph value (4.5, 5.0, 5.5 and 6.0) . Different sludge percentage values (2.5, 5, 7.5 and 10%)	5.988 ± 0.5 L H_2/L-med 10% POME sludge (w/v) pH 5.5	Norfadilah *et al.*, 2016
Biohydrogen production from palm oil mill effluent using immobilized cells	. *Clostridium butyricum* EB6 . Polyethylene glycol (to immobilize cells)	510 mL H_2/L-POME h	Singh *et al.*, 2013
Biohydrogen production from anaerobically treated POME in bioreactor under optimized condition	. Mesophilic conditions . pH control . 7 hours of fermentation	Hydrogen yield (Ps): 1.32 L/L POME Hydrogen production rate (Rm): 0.144 L/L.h	Rasdi *et al.*, 2012

These studies showed the use of different types of bioreactors, empirical models of hydrogen production process, use of microflora and others methods. Each result is good and scientifically interesting (Singh *et al.,* 2013).

Hydrolysis of POME and the liberation of fermentable sugars in the process, according to Azman (2016), made possible the use of this wastewater as a substrate for the biohydrogen production. However, improvement conditions were obtained with the control and interaction between the incubation temperature and the amount of inoculum.

According to Krishnam (2016), the production of biogas (hydrogen and methane) in two-stage process is viable and has the capacity to remove organic matter from of the fermented. The recirculation of sludge between the reactors is another positive factor of this process.

Norfadilah (2016) concluded that pH control improves biohydrogen production with a dilute amount of POME sludge (about 10%). In addition, COD reduction was also significant. At the end of fermentation, the hydrogen production efficiency is 62.25%.

The use of immobilized cells, according to Singh *et al.,* (2013), has proven to be an excellent alternative for the improvement of the hydrogen production process in wastewater, such as POME. It was also observed the reduction of the organic matter for the treatment of the fermented.

Rasdi (2012) showed favorable results for the use of POME in the biohydrogen production, with the control of parameters such as pH and COD. It has been found that pH control accelerates gas production and can be a factor of process improvement.

3 MATERIALS AND METHODS

3.1 POME CHARACTERIZATION

Three different samples from a palm oil industry, Biopalma (Mojú, Pará - Brazil) were stored and classified in different forms: P001 was maintained in a tank under room temperature, P002 was frozen and P003 was cooled at 4ºC. The samples characterization after storage is presented in Table 7.

3.2 POME PREPARATION

The media were prepared according to Sydney (2013) with modifications. POME (raw, diluted or hydrolyzed POME) was supplemented with 1% (w/v) of sucrose. The pH of the culture media was adjusted to 7.0 with 1N KOH. The media were boiled under slight stirring and degassed with carbon dioxide. The gas passed through the POME media cooled it, and at 85ºC it was added 0.5% of sodium bicarbonate; at 65ºC was added 0.01% of L-cysteine. Hungate tubes were filled with 5mL of POME media, degassed with carbon dioxide, sealed to avoid the presence of oxygen with Bakelite screw caps and rubber stoppers and autoclaved.

Different POME media were prepared , The diluted POME was prepared with water (1:2), and the hydrolyzed POME was done according to Azman *et al.*, (2016). These procedures were done with both POME samples (P001 and P003).

3.3 MICROORGANISMS AND INOCULUM

Microbial consortia were previously selected from earlier studies (Sydney, 2013) and cultivated in vinasse anaerobic medium. The viability of the inoculum was evaluated by measuring the volume of gas produced using glass injection syringe.

Eleven available consortia were inoculated in anaerobic POME media in a proportion of 1mL of inoculum to 5 mL of media, and incubated at 37ºC for seven days. After the fermentation period, five consortia and a single strain (ATCC 8260) were considered adapted to POME. The name and origin of the consortia are presented in Table 6, as well as the ATCC strain previously selected as biohydrogen producer.

Table 6: Selected consortia (and strain) for biohydrogen production in POME.

Code	Name according to Sydney (2013)	Origin
C3	LPB AH3	Soil used for Sugarcane cultivation
C5	ATCC 8260	LPB strain bank – *Clostridium beijerinckii*
C6	LPB AH8	Vinasse pond
C9	LPB AH9	Clermont University strain bank
C10	LPB AH1	Faeces from fruit bat (unknown species)
C12	LPB AH2	Liquid waste lake of a dairy farm

Source: The author (2017).

Two other tests were made to select the microorganisms: 1) The POME media without autoclaving was subject the same conditions of fermentation and 2) three generations were done with vinasse consortia in POME for adaptation of microorganism. The results weren't significant in both tests. The trials followed with selected consortia and strain.

3.4 MICROBIAL CONSORTIA IDENTIFICATION

Approximately 5 mL of each consortia sample were processed for DNA extraction with phenol / chloroform, followed by the PCR analysis for the V4 region of the 16S rDNA gene with 10ng of DNA, primers 515F and 806R, and the KlenTaq system (Sigma-Aldrich, USA) according to the methodology of Caporaso *et al.* , (2012). The thermocycling consisted of 96ºC for 3 minutes, 18 cycles of 96°C for 20 seconds, 50°C for 45 seconds and 68°C for 1 minute. The resulting amplicons were analyzed by electrophoresis with 1.5% agarose gel and quantified with the Qubit kit (Invitrogen). The amplicons were diluted to 16pM and sequenced on the Illumina MiSeq platform with the 500V2 set, which generated 250bp readings. As sequences generated with the Qiime program, using as cut line 16000 readings / sample with the Silva database with 97% identity.

3.5 ISOLATION OF BACTERIA FROM THE CONSORTIA

The isolation of the microorganisms from the consortia was performed aiming to test them as biohydrogen producers. A sample of each consortium was placed in MRS and Clostridium media. The MRS medium was prepared (per liter) with 10g of

peptone, 10g of beef extract, 5g of yeast extract, 20g of dextrose, 2g of ammonium citrate, 5g of sodium acetate, 0.1g of magnesium sulfate, 0.05g of manganese sulfate, 2g of dipotassium phosphate and 15g of bacteriologic agar. The *Clostridium* medium was prepared (per liter) with 10g of tryptose, 10g of beef extract, 3g of yeast extract, 5g of dextrose, 5g of sodium chloride, 1g of soluble starch, 0.5g of L-cysteine, 3g of sodium acetate and 15g of bacteriologic agar. The isolation was performed in three steps: vinasse consortia were inoculated in anaerobic MRS and *Clostridium* broth media (without agar), this inoculum was striated in Petri dishes with the same media (incubated for 7 days at 37ºC in an atmosphere by anaerobic kit in the jar) and the different colonies formed were inoculated again into sterilized broth media in Hungate tubes (both MRS and *Clostridium* medium respectively). Gram staining was done during the three steps to check the bacteria isolation. Finally, two species of bacteria were isolated of each medium and consortia. The biohydrogen production was used to select the best isolates to follow with the experiments.

3.6 GROWTH KINETIC OF MICROORGANISMS

Analysis of the growth of consortia, pure strain and isolated bacteria was done to determine the inoculum volume necessary to produce biohydrogen with high yield. Firstly, the microorganisms were inoculated in new anaerobic sterilized POME medium. The growth was evaluated daily for 8 days. The absorbance was measured at 540 nm using a spectrophotometer (optical density). From the results, the time to reach the maximum concentration of cells and the inoculum volume for fermentation was determined, according to Table 9.

3.7 BIOHYDROGEN PRODUCTION

An experimental design was used to improve the production of biohydrogen. Firstly, the consortia, isolated bacteria and pure strain were cultivated in POME using the Sydney (2013) method, and compared for the gas production. The best combinations of POME and microorganisms were selected using the Tukey Test. These combinations were evaluated using a Plackett-Burman design, with 7 factors and 8 runs. The three main (significative) factors for biohydrogen production were used in a complete factorial design, with 3 factors, 8 runs and triplicate at the central point.

The biohydrogen content was measured by gas chromatography (Agilent 490 MicroGC, Agilent Technologies) equipped with a MolSieve 5A and a Pora PLOT U columns. The columns operated at 90ºC. Argon was used as carrier gas at 200kPa and 150kPa, respectively for each column. The injector was maintained at 110 ºC, the stabilization time was 5s, 30s of sample time, 30ºC of sample temperature, 40ms of injector time, and 11s of backflush for the analysis with the MolSieve 5A and and 14s of backflush for the other column. The samples were injected in the MicroGC with a glass syringe.

A gas mixture of 20.04% mol/mol of methane, 19.95% mol/mol of carbon dioxide, 10.05% mol/mol of hydrogen and nitrogen for the balance, was used as standard. This gas was injected in microGC and the peak area was related with the biohydrogen production of each sample. The biohydrogen volume calculation was done with the Eq. [5].

Biohydrogen volume (mL)= (%molH_2/mol * biogas volume (mL))/100000 [5] (The author, 2017)

The % molH_2/mol was obtained from the peak area (in mV.min) ratio during the analysis of the chromatograms for cultures in 5 ml of medium.

3.8 ANALYSIS OF VOLATILE FATTY ACIDS (VFAS) AND SUGAR CONSUMPTION

The fermented material was analyzed at the end of the fermentation process. The volatile fatty acids were measured by High Performance Liquid Chromatograph (HPLC) equipped with a Aminex® HPX-87H 300 x 7.8mm (Bio-Rad) column and a refractive index detector (RID-10A). The column was kept at 60^{o}C and 5mM H_2SO_4 at 0.6 mL/min was used as mobile phase and 20µL of sample injection volume. A refractive index detector was used and maintained at 40ºC. The analysis measured the concentration of succinic, lactic, formic, acetic, propionic and butyric acids in g/L (Sydney 2013). The sugar consumption was measured using the DNS method according to Libardi *et al.*, (2017), where the samples was measured by microplate reader BioTek Powerwave XS.

4 RESULTS AND DISCUSSION

4.1 PHYSIOCHEMICAL ANALYSIS OF POME

The results of physicochemical characteristics of POME are in Table 7. The main parameters were compared with the fresh POME (when they were collected in the industry), such as COD and oil and grease.

Table 7: Physicochemical analysis of POME samples.

Parameters	**POME Storage Conditions**			
	Fresh POME	**Room temperature**	**Frozen**	**Cooled**
		P001	**P002**	**P003**
Biochemical oxygen demand (BOD_5) - mgO_2/L	22158	34771	35719	52676
Soluble biochemical oxygen demand (BOD_5) - mgO_2/L	10519	14270	18729	14677
Chemical oxygen demand (COD) - mgO_2/L	74908	89591	97958	85714
Soluble chemical oxygen demand (COD) - mgO_2/L	18041	22653	17959	60816
Total dissolved solids (TDS) (mg/L)	9250	9310	7410	12310
Total suspended solids (TSS) (mg/L)	53385	36560	27750	56760
Total solids (TS) (mg/L)	62794	47050	43785	64680
Oil and grease (mg/L)	20703	37883	39249	30209
pH	4.32	4.63	4.44	4.31
F^- (mg/L)	-	0	-	8340
Cl^- (mg/L)	-	113.74	-	94.07
Br^- (mg/L)	-	21.48	-	18.26
NO_3^- (mg/L)	-	0	-	0
PO_4 (mg/L)	-	0	-	0
SO_4^{-2} (mg/L)	-	33.16	-	32.46
Na^+ (mg/L)	-	27.50	-	318.20
NH_4^+ (mg/L)	-	329.25	-	0
K^+ (mg/L)	-	2363.78	-	1331. 00
Mg^{+2} (mg/L)	-	326.89	-	260.65
Ca^{+2} (mg/L)	-	309.59	-	242.69
Reducing sugar concentration (mg/L)	-	228	-	236

Source: The author (2017).

The difference observed in the parameters analyzed of the POME samples (COD (74908mgO_2/L) and oil and grease (20703mg/L)) was attributed to the storage conditions and it was determined for the selection of the representative samples P001 and P003.

Based on results from characterization and data from other articles, it was decided to use the POME in three different forms: raw POME, diluted POME and hydrolyzed POME. Diluted and hydrolyzed modifications of the POME media were done, to investigate 1) if the dilution of the high organic load of the medium would stimulate cell growth and 2) if hydrolyzed media would increase sugars availability in the medium. The high organic load is relevant to the process, mainly characteristic of POME but may inhibit microbial growth during fermentation. So it was necessary to test new culture media.

The amount of reducing sugars in POME was low (between 228 and 236 mg/L), which led to the addition of this compound to the culture medium. According to Sydney (2013), the selected consortia needed 10g/L of fermented sugar in the medium to produce biohydrogen. Considering the content of sugar in the POME and the storage time (24 months), it was decided to add the half of the concentration (5g/L).

4.2 CONSORTIA IDENTIFICATION AND MICROORGANISM ISOLATION

The five consortia and the C5 strain were identified and presented in Table 8. The strain C5 was subjected to identification due to possible previous contamination, which was proved to be the case, because only 97.43 of the microorganisms were *Clostridium.*

Table 8: Identification of consortia C3, C6, C9, C10 and C12 and the *Clostridium beijerinckii* C5.

CONSORTIA/ STRAIN	**FAMILY/ GENUS IDENTIFICATION**	**% OF FAMILY/ GENUS IN THE SAMPLE**
C3	*Sporolactobacillus*	96.22
	Clostridium	2.65
	Clostridiaceae (no genus defined)	0.97
	Other genus	0.16

C5	*Clostridium*	97.43
	Oxalobacteraceae (no genus defined)	1.11
	Lactobacillus	1.07
	Other genus	0.39
C6	*Lachnospiraceae (no genus defined)*	85.72
	Clostridium	9.04
	Ruminococcus	4.87
	Other genus	0.37
C9	*Sporolactobacillus*	99.65
	Other genus	0.32
	Clostridiaceae (no genus defined)	0.03
C10	*Oxalobacteraceae (no genus defined)*	52.23
	Lactobacillus	24.29
	Other genus	4.55
	Ruminococcus	3.96
	Brucellaceae (no genus defined)	2.98
	Enterobacteriaceae (no genus defined)	2.85
	Bartonellaceae (no genus defined)	1.69
	Pseudomonas	1.64
	Acinetobacter	1.53
	Providencia	1.34
	Lachnospiraceae (no genus defined)	1.25
	Stenotrophomonas	1.16
	Agrobacterium	0.53
C12	*Oxalobacteraceae (no genus defined)*	49.55
	Lactobacillus	19.71
	Clostridium	9.7
	Other genus	5.66
	Brucellaceae (no gender defined)	2.9
	Pseudomonas	2.7
	Enterobacteriaceae (no genus defined)	2.53
	Bartonellaceae (no genus defined)	1.65
	Providencia	1.4
	Acinetobacter	1.2
	Erwinia	0.8
	Stenotrophomonas	0.6
	Agrobacterium	0.54
	Burkholderia	0.4
	Bacillus	0.4

	Sphingomonas	0.2
	Rhizobium	0.05
	Desulfosporosinus	0.01

Source: The author (2017).

The microscopic analysis with Gram staining showed the presence of bacterial forms present in the consortia. All isolated samples formed, on average, two distinct colonies, which were mostly *Lactobacillus* and *Clostridium* genus. The result of morphology of each colony of bacteria was compared with the consortia identification, with the bacteria known as hydrogen producers in the literature and with the microorganisms found in the regions of origin of the consortia and the isolation was made in selective medium MRS (for *Lactobacillus*) and *Clostridium* medium. From the C3 consortium was isolated in MRS medium, a rod-shaped Gram-positive bacteria, possibly the *Sporolactobacillus* (Singh and Wahid, 2015). From the C5 strain sample, a Gram-positive rod-shaped bacterium was isolated in MRS and Clostridia media, consistent with the *C. beinkerinckii* ATCC 8260 (Pan *et al.*, 2008). From the C6 consortium it was isolated a Gram-positive bacterium, and butyric acid producer (5.85g/L on average), probably belonging to the *Lachnospiraceae* family, (the molecular analysis of this consortium did not reached the genus level). From the C9 consortium it was isolated, in MRS medium, a bacterium with the same characteristics of the isolate from C3 - possibly *Sporolactobacillus* of another species due to low hydrogen production. From the C10 consortium, it was isolated a bacteria possibly belonging to the *Oxalobacteraceae* family, due to the low biohydrogen production, the prevalence of this family in microbial consortium identification and the absence of the main source of carbon (oxalate) in POME (Chapelle *et al.*, 2016). The same conclusion was reached with the analyzes of the C12 consortium, that is, it should be bacteria of the *Oxalobacteraceae* family (Sarma *et al.*, 2012).

The considerable presence of *Lactobacillus* and *Clostridium* in almost all the consortia justifies the production of biohydrogen. However, with an addition of sucrose to increase the concentration of sugars in the medium, the consortia with a higher proportion of *Clostridium* gave a greater production of hydrogen, when compared to *Lactobacillus* due to the production of lipases responsible for the hydrolysis of lipids present in POME (Guo *et al.*, 2010).

4.3 BIOHYDROGEN PRODUCTION

4.3.1 Growth kinect of microrganisms

With the results of optical density analysis, it was possible to estimate the fermentation time in which there is the maximum bacterial growth, according to Table 9.

Table 9: Fermentation time (in days) estimated by bacterial growth

CODE OF MICRORGANISM	CONSORTIA	ISOLATED BACTERIA
C3	4	4
C5	4	4
C6	6	6
C9	6	6
C10	4	4
C12	4	4

Source: The author (2017).

4.3.2 Experimental Planning

A first screening of microorganism and POME media were done to evaluate the gas production (Table 10). The highest production of gas was obtained in Raw P001, Raw P003 and Diluted P001 with C6 consortia. With the C5 strain (ATCC 8260), the best production was reached with Diluted P003, Hydrolyzed P001 and Hydrolyzed P003. The Tukey tests was done to prove what was already observed in the first screening: a low productivity of gas in Raw P001, which was discarded.

Table 10: First screening results with the Average volume of biogas production by the selected microbial consortia and strain in different POME media.

CODE	A. RAW POME 001	B. RAW POME 003	C. DILUTED POME 001	D. DILUTED POME 003	E. HYDROLYSED POME 001	F. HYDROLYSED POME 003
	Average volume (mL)					
C3	0	12	0.33	5.33	2.67	15.33
C5	0	10.67	2.33	11.67	19.33	16.67
C6	5.33	15	19.33	10.67	2	12
C9	0.67	19.33	0	6.33	1.33	8.33
C10	0	6.67	4.33	0	14.67	0
C12	0	9.33	1.67	0	0	3.66

Source: The author (2017).

An experimental Plackett Burman planning was performed to evaluate the influence of seven main factors of the biohydrogen production: sucrose, temperature, pH, time of fermentation, L-cysteine concentration, inoculum volume and type of consortia (or strain) were tested at different conditions totaling eight runs. This test was analyzed using $p<0.05$ of significance and selected the sucrose concentration, fermentation time, temperature and inoculum volume as influent factors of gas production (Table 11).

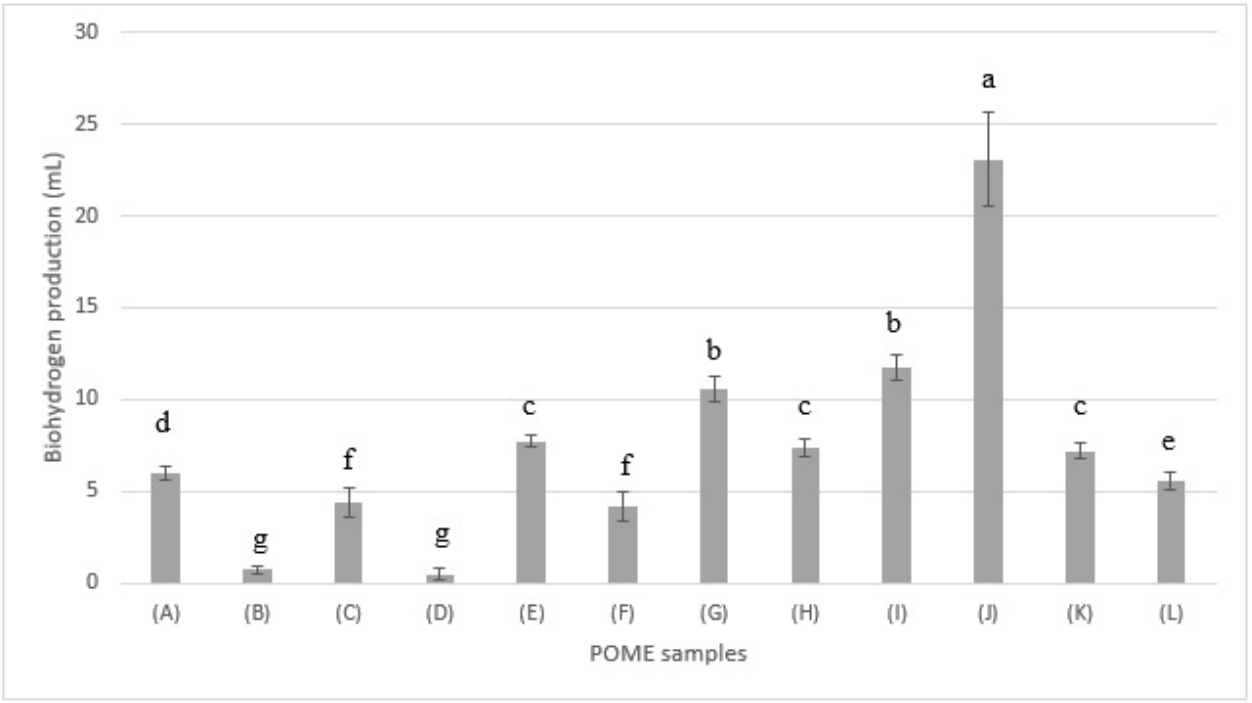
Table 11: Results of biogas production in different POME media and conditions according to the Plackett Burman test. In the last column of type of consortia, the Raw P003 variation (-1) is C6 consortia and (1) is C9 consortia; the Diluted P003 variation (-1) is C6 consortia and (1) is C5 (the ATCC strain); the Hydrolyzed P001 variation (-1) is C10 consortia and (1) is C5 (strain) and the Hydrolyzed P003 variation (-1) is C3 consortia and (1) is C5 (strain). As the best results of Diluted P001 were with C6 consortia, the last factor was changed for peptone concentration. In this case, the variation (-1) is zero and the (1) is 10g/L of peptone.

Run	Run conditions							Biogas production(mL)				
	Sucrose (g/L)	Temp. (°C)	pH	Time (days)	L-cysteine (g/L)	Inoculum volume (mL)	Consortia (or strain)/ Peptone (g/L)	Raw P003	Diluted P001	Diluted P003	Hydrol. P001	Hydrol. P003
1	0	30	6	6	1	1	-1	8	0	6	0	16
2	5	30	6	4	0	1	1	0	0	20	13	28
3	0	37	6	4	1	0.5	1	0	0	0	0	0
4	5	37	6	6	0	0.5	-1	16	4	12	4	20
5	0	30,0	7	6	0	0.5	1	0	3	4	4	20
6	5	30,0	7	4	1	0.5	-1	12	4	12	0	16
7	0	37,0	7	4	0	1	-1	5	3	3	0	16
8	5	37,0	7	6	1	1	1	8	6	18	16	28

Source: The author (2017).

In the Complete Factorial test, the C6 consortium was cultivated in Raw P003 and Diluted P001; in Diluted P003 and Hydrolyzed P001 was inoculated C5 in vinasse and in Hydrolyzed P003, C5 in MRS medium (isolated *Clostridium beijerinkii*). The biohydrogen production results in different POME media and consortia C3, C6, the strain C5 and their isolated forms are in Graphic 1.

Graphic 1: Biohydrogen production results in duplicate (A) Raw P003 with C6 consortium, (B) Raw P003 with *Lacnospiraceae* isolated from C6, (C) Diluted P001 with C6 consortium, (D) Diluted P001 with *Lacnospiraceae* isolated from C6, (E) Diluted P003 with C5, (F) Diluted P003 with *Clostridium beijerinckii,* (G) Hydrolyzed P001 with C5, (H) Hydrolyzed P001 with *Clostridium beijerinckii,* (I) Hydrolyzed P003 with C5, (J) Hydrolyzed P003 with *Clostridium beijerinckii,* (K) Hydrolyzed P003 with C3 consortium, (L) Hydrolyzed P003 with *Sporolactobacillus* isolated from C3.



Source: The author (2017).

The highest biohydrogen production was reached with *Clostridium beijerinckii* cultivated in Hydrolyzed P003 (approximately 23ml). It is possible to verify that samples of Diluted P003 and Hydrolyzed P001 also had a good result, and although all samples were made with the inoculum of C5 in vinasse, both were chosen for the scale change of fermentation. The C3 consortium produced the best results in its raw form, showing that consortia with *Sporolactobacillus* are good producers of biohydrogen, because they are similar to *Lactobacillus* in metabolism even though it is strictly anaerobic (Margulis and Chapman, 2009). However, the ability to ferment substrates for the formation of lactic acid limits the production of hydrogen (De Vos *et al.*, 2009).

The C6 consortium in Raw P003 also presented significant results, although low compared to Hydrolyzed P003 with *C. beijerinckii.* The bacteria of the *Lachnospiraceae* family from the order *Clostridiales* are potential producers of

hydrogen in raw POME and this is quite feasible in economic terms due to the use of the effluent without pre-treatments or dilutions (Norfadilah *et al.*, 2016).

4.3.3 Scaled up biohydrogen production

With the results of the experimental planning, a scale up was done with the best samples, where they were observed besides the production of hydrogen, the concentration of volatile fatty acids and the sugar consumption (Table 12). The maximum yield of hydrogen was obtained with Hydrolyzed P003 which, although it did not produce acetic acid in large quantities, had this as the main fermentation product, where the gas was produced from the reduction of NADH to NAD + (Júnior *et al.*, 2014). In the samples of Diluted P003 and Hydrolyzed P001, there was a high production of both acetic acid and butyric acid (where NADH is used for the oxidation of Acetyl CoA to butyrate) and this possibly affected the efficiency in the production of hydrogen. In addition, both produced lactic acid and propionic acid in reasonable quantities, which also reduces the yield of the process (Chin *et al.*, 2003). the consumption of sugar showed that the addition of sucrose in the fermentation process was important for bacterial growth, which resulted in a production of biohydrogen close to other articles.

Three of the best results of biohydrogen production were scaled up to evaluate the hydrogen production in 1L anaerobic reactors (Fig. 3). Diluted P003 was inoculated with 100mL of the C5, supplemented with sucrose to 5g/L, and incubated at 30ºC. Hydrolyzed P001was inoculated with 100mL of the C5 and added 9.2g/L of sucrose and 6 days of time and Hydrolyzed P003 was inoculated with 134mL of C5 isolated bacteria and added 7.5g/L of sucrose and 8 days of time. The hydrogen production in 1L bottles is presented in Table 12.

Figure 3: 1L bottle used to hydrogen production with POME Diluted P003, Hydrolyzed P001 and Hydrolyzed P003 media at the same conditions of the tube.

Source: The author (2017).

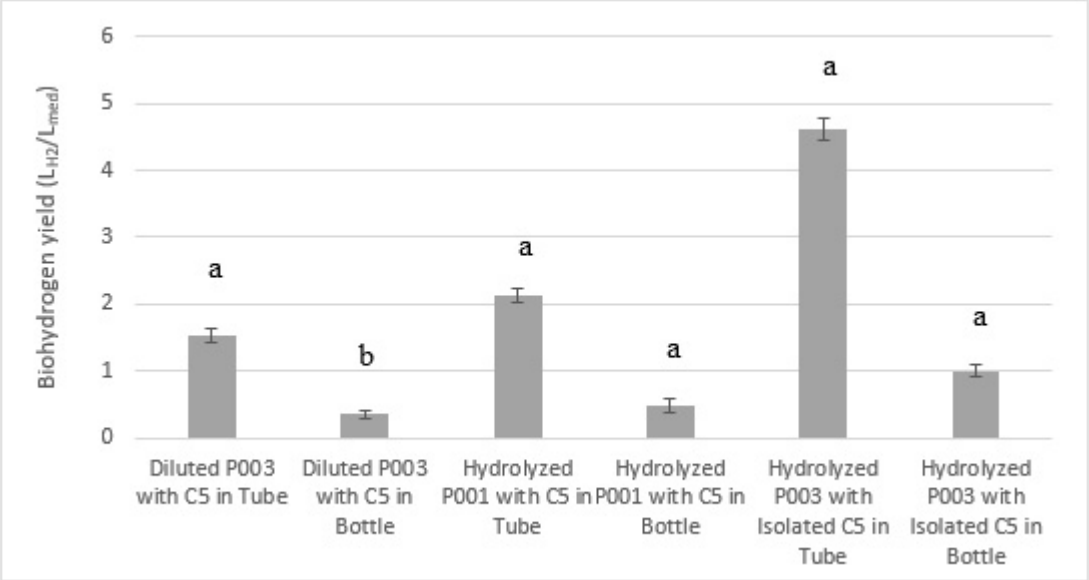
Table 12: Results of biohydrogen production in tubes and bottles.

POME MEDIA	INOCULUM	FLASK	$BIOH_2$ VOLUME (L)	$BIOH_2$/ MEDIUM (LH_2/L_{med})	Volatile fatty acids (g/L)						SUGAR COMSUMPTION (g/L)
					acetic	propionic	butyric	succinic	lactic	formic	
Diluted P003	C5	Tube	0.0077	1.531	3.520	1.242	5.026	0.209	0.818	0.819	4.381
		Bottle	0.1184	0.355	4.823	1.422	5.118	0.217	0.990	0.801	4.421
Hydrolyzed P001	C5	Tube	0.0106	2.122	2.842	1.671	3.098	0.508	0.567	0	6.513
		Bottle	0.1619	0.486	5.312	2.166	4.685	0.103	0.363	0.717	6.612
Hydrolyzed P003	isolated C5	Tube	0.0231	4.620	1.519	1.038	0	0.735	0	0	7.020
		Bottle	0.3345	1.004	1.571	1.085	0	0.718	0	0	7.072

Source: The author (2017).

The final results of the biohydrogen production are compared in the Graphic 2.

Graphic 2: Biohydrogen production yield by *Clostridium beijerinckii* in POME at different fermentation conditions in tubes and bottles in duplicate with p<0.01 according to Tukey Test.



Source: The author (2017).

The Hydrolyzed P003 with isolated C5 strain showed the best biohydrogen production yield 4.620LH_2/L_{med} in tubes and 1.004LH_2/L_{med} in bottles. The *Clostridium beijerinckii* strain showed a good result, similar to that of other researches with the same type of substrate, POME: 5.988 LH_2/L_{med} by Norfadilah *et al.*, (2016), and 5.350 LH_2/L_{med} by Singh *et al.*, (2013). The same strain when cultivated in vinasse based medium produced 4.441. LH_2/L_{med}. (Sydney, 2013). Although the volume of gas produced increased in the bottle approximately 15 times, the yield was 21% of what was obtained in tubes, following the same medium / bottle ratio. The bakelite caps of the tubes promoted a much greater control of the seal than in the bottle, which had inputs of materials and outlets for the gas produced. Several leak tests have been done (foam, submersion and others), but hydrogen is very light. In addition, measurements of the gas produced in tubes were made once by a 100ml glass syringe, whereas in the bottles, the measurements were made in batch, using a valve to limit the outflow of the gas.

Clostridium beijerinckii is well known for its ability to use different carbon sources to produce hydrogen as well as its potential to convert effluents into metabolites of interest. This strain produced more hydrogen compared to other consortia (Liu *et al.*, 2016). Its isolated or contamination-free form gave an even higher hydrogen yield when compared to C5 in vinasse, possibly because of the

composition of the selective medium MRS, rich in several nutrients, especially potassium diphosphate. This nutrient acts as a buffer that controls the acidification of the medium throughout the fermentation process, which prevents the pH reduction that would affect the efficiency of the process (Pan *et al.*, 2008). Consequently, the hydrogen production becomes larger, as is seen in the samples with the Hydrolyzed P003 medium.

Among the main control parameters in the hydrogen production is the pH, which in the case of the C5 strain is around 6.0 to 7.0. Diluted P003 produced the least amount of hydrogen and pH 6 (limit) may have affected the activity of the hydrogenase enzyme, responsible for the production of hydrogen, as well as having altered the metabolic pathway (Trchounian *et al.*, 2017).

The fermentation time was pre-determined from the growth kinetic of the bacteria in the different types of culture medium. Despite this, better results were obtained in higher fermentation time, as in the Hydrolyzed P003 sample with 8 days of fermentation. With a higher concentration of sugars, the cell growth had a longer duration, as did the exponential phase (Jung *et al.*, 2011).

Despite the high gas pressure inside the Hungate tubes, it was found that a daily measurement did not yield good results and the method of measuring the biogas produced (by detachment from the syringe plunger) was not effective. It required a buildup of gas and consequently an increase in pressure, so that there was an efficient evaluation of production.

The consumption of sugars by the hydrogen producing bacteria also followed the biohydrogen prodution results, and in Diluted P003 it was approximately 84% in tube and 85% in bottle, Hydrolyzed P001 68% and 69% and Hydrolyzed P003, 89% in both. This shows that the addition of sucrose really was necessary for the increase of hydrogen production by strain C5 (*Clostridium beijerinckii*).

5 CONCLUSION AND FUTURE PERSPECTIVES

Mojú's samples of POME showed to be a good substrate for the biohydrogen production, despite the storage time. Higher yield of H2 was obtained with *Clostridium beijerinckii* (ATCC 8260) conserved in MRS medium and cultivated in hydrolyzed POME (P003). Other bacteria and consortia are good producers of hydrogen, such as C3 and C6 (Graphic 1), but they depend on an adequate control of the parameters that most interfere in the fermentation.

In addition, POME has a potential producer of hydrogen in various forms (pure, diluted and hydrolyzed) and may be increased not only by sucrose but also by other sugars. Hydrolyzed P003 showed better results due to the adequate concentration of sugar (obtained by hydrolysis and added), pH (around 6.0 to 7.0), fermentation time (8 days) and formation of acetic acid (1.519 g/L) during fermentation. Theabsence of acids that could direct the metabolic pathway to another product, such as lactate, also indicate the hydrolyzed P003 as a good medium to hydrogen production. The best results were with the experiments in the tubes due to the lower volume of the flask and better control condition.

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