UNIVERSIDADE DE CAXIAS DO SUL
ÁREA DO CONHECIMENTO DE CIÊNCIAS DA VIDA

INSTITUTO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

Identificação e caracterização de consórcios microbianos produtores de hidrogênio e outros coprodutos a partir de vinhaça de cana-de-açúcar, após pré-tratamento do inóculo

Flaviane Eva Magrini

Caxias do Sul
2019

Flaviane Eva Magrini

Identificação e caracterização de consórcios microbianos produtores de hidrogênio e outros coprodutos a partir de vinhaça de cana-de-açúcar, após pré-tratamento do inóculo

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção de grau de Doutor em Biotecnologia**.**

Orientadora: Prof. Dra. Suelen Paesi
Co-orientador: Prof. Dr. Maurício Moura da Silveira

Caxias do Sul
2019

Dados Internacionais de Catalogação na Publicação (CIP)
Universidade de Caxias do Sul
Sistema de Bibliotecas UCS - Processamento Técnico

M212i Magrini, Flaviane Eva

Identificação e caracterização de consórcios microbianos produtores de hidrogênio e outros coprodutos a partir de vinhaça de cana-de-açúcar, após pré-tratamento do inóculo / Flaviane Eva Magrini. – 2019.

122 f. : il. ; 30 cm

Tese (Doutorado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Biotecnologia, 2019.

Orientação: Suelen Osmarina Paesi.

Coorientação: Mauricio Moura da Silveira.

1. Hidrogênio como combustível. 2. Resíduos indústriais. 3. Resíduos agrícolas. 4. Cana-de-açúcar. 5. Vinhaça. I. Paesi, Suelen Osmarina, orient. II. Silveira, Mauricio Moura da, coorient. III. Título.

CDU 2. ed.: 662.769.2

Catalogação na fonte elaborada pela(o) bibliotecária(o)
Ana Guimarães Pereira - CRB 10/1460

Flaviane Eva Magrini

IDENTIFICAÇÃO E CARACTERIZAÇÃO DE CONSÓRCIOS MICROBIANOS PRODUTORES DE HIDROGÊNIO E OUTROS COPRODUTOS A PARTIR DE VINHAÇA DE CANA-DE-AÇÚCAR, APÓS PRÉ-TRATAMENTO DO INÓCULO

Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção do título de Doutora em Biotecnologia.

Orientadora: Profa. Dra. Suelen Osmarina Paesi

Co-orientador: Prof. Dr. Maurício Moura da Silveira

TESE APROVADA EM 23 DE AGOSTO DE 2019.

_____

Orientadora: Profa. Dra. Suelen Osmarina Paesi

_____

Co-orientador: Prof. Dr. Maurício Moura da Silveira

_____

Prof. Dr. Aldo José Pinheiro Dillon

_____

Profa. Dra. Simone Damasceno Gomes

_____

Profa. Dra. Valéria Reginatto Spiller

Dedico ao meu filho Érico, quem me ensinou a ver o mundo com outros olhos, me mostrou diferentes caminhos a serem seguidos e principalmente a nunca desistir!

AGRADECIMENTOS

Primeiramente gostaria de agradecer a Deus, em todas as suas formas de manifestações, pelos caminhos que me trouxeram até aqui.

A minha família, pela compreensão nas ausências nos encontros familiares. Em especial ao meu amor Eduardo Vanelli, que foi meu maior incentivador, que mesmo nos momentos de maiores dificuldades, nunca deixou que eu desistisse. Ao meu filho Érico, a paixão da minha vida, que chegou em meio ao doutorado, me mostrando um caminho azul que eu jamais imaginei que existisse. Ensinou-me que a persistência é o caminho para o êxito.

A minha orientadora Prof. Dra Suelen Paesi, pelas oportunidades no Laboratório de Diagnóstico Molecular, pela orientação, incentivo, confiança, preocupação e pela amizade que conquistamos em todos estes anos de convívio.

Aos colegas do laboratório, Janaina Iltchenco, Liliane Poleto, Ariela Schiavenin, Andressa Castilhos, Ana Silvia Eder, pela parceria, amizade e muita troca de artigos.

Aos colegas e bolsistas de iniciação científica do laboratório, Mariana Smiderle, Luana Lora, Valéria dal Prá, Andressa Spengler, Leandro Gois de Almeida, Júlia Tonioli, Darliane Andreis, pela parceria e muita risada. Aos alunos Denis da Maia Soares e Gabriela Machado de Almeida pela valiosa ajuda nos inúmeros ensaios realizados neste trabalho.

Ao Prof. Dr. Lademir Luiz Beal, pela colaboração com o projeto de pesquisa e empréstimo de equipamentos no LATAM. As funcionárias e amigas Juliane Azevedo e Edinéia Stroeher pela ajuda com as análises das amostras.

A Prof. Dra Claudia Ecthebehere e Laura Fuentes, pelo acolhimento e ensinamento no Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguai.

Ao Prof. Dr. Mauricio Moura da Silveira, pela co-orientação e auxílio nos cálculos. Aos professores membros da banca de acompanhamento Dr. Aldo Dillon e Dra. Marli Camassola, pelas importantes contribuições neste trabalho.

A todos os amigos e funcionários dos demais laboratórios e secretarias do Instituto de Biotecnologia, obrigada pela amizade.

A Universidade de Caxias do Sul e ao Programa de Pós Graduação em Biotecnologia. A Petrobrás pelo financiamento deste projeto.

SUMÁRIO

LISTA DE TABELAS

Lista de tabelas do Capítulo 1 – Artigo 1

Lista de tabelas do Capítulo 2 – Artigo 2

LISTA DE FIGURAS

Lista de figuras do Capítulo 1 – Artigo 1

Lista de figuras do Capítulo 2 – Artigo 2

LISTA DE SÍMBOLOS

°C – graus Celsius

min – minuto

mL – mililitr

mol – mol

mmol - milimol

g – grama

L - litro

KJ – kilojoule

mm – milímetros

µL – microlitro

h- hora

v – volume

p - pressão

nm - nanômetros

M - molar

LISTA DE ABREVIATURAS

AGV – ácidos graxos voláteis

CO – monóxido de carbono

CO_2 – dióxido de carbono

DQO – demanda química de oxigênio

COD – *chemical oxigen demand*

SSV – sólidos suspensos voláteis

DGGE – *desnaturing gradiente gel eletrophoresis*

CH_4 - metano

DNA – *deoxyribonucleic acid*

F – forward

R - reverse

H_2 – gás hidrogênio

NAD – coenzima nicotinamida adenina dinucleotídeo oxidada

NADH – coenzima nicotinamida adenina dinucleotídeo reduzida

OTU – unidade taxonômica operacional

P – volume cumulativo de H_2

PCR – *polymerase chain reaction*

pH - potencial hidrogeniônico

R*m* – velocidade máxima de produção de hidrogênio

rRNA – ácido ribonucleico ribossomal

RT-PCR – *real time PCR*

VFA – *volatile fatty acids*

T90 – tratamento térmico do inóculo em 90°C por 10 min

T105 - tratamento térmico do inóculo em 105°C por 120 min

T121 - tratamento térmico do inóculo em 121°C por 20 min

T1 – T90 pH 6

T2 – T105 pH 5

T3 – T121 pH 6

RESUMO

A utilização de hidrogênio como combustível é considerada uma alternativa promissora para as próximas décadas, por ser uma fonte limpa de energia e produzir apenas água como produto de sua combustão. A produção biológica de hidrogênio pode ser realizada utilizando diversos resíduos agroindustriais. Entre estes resíduos encontra-se a vinhaça que é proveniente da produção de etanol, sendo gerado em proporções de 12 a 15 litros para cada litro de etanol produzido. Atualmente, a vinhaça é utilizada principalmente como fertilizante, no próprio cultivo da cana-de-açúcar, no entanto, os órgãos ambientais estão limitando a quantidade de vinhaça que pode ser aplicada ao solo devido ao seu alto poder poluente. O processo de conversão da vinhaça em hidrogênio pode ser realizado utilizando culturas puras ou consórcios microbianos. O uso de consórcios pode ser mais vantajoso, pois são mais simples de manusear e não requer meios estéreis. No entanto, alguns microrganismos contidos nos consórcios podem ser consumidores de H_2, o que torna necessário submeter o inóculo a um pré-tratamento para suprimir a atividade destas bactérias. Dessa forma, o objetivo deste trabalho foi avaliar três métodos de pré-tratamento térmico do inóculo para a produção de hidrogênio e ácidos graxos voláteis a diferentes valores iniciais de pH, utilizando a vinhaça como substrato e, comparar a composição da comunidade microbiana. Os ensaios para a produção de H_2 foram realizados utilizando vinhaça a 37°C, com os seguintes pré-tratamentos do inóculo: 90°C/10min; 105°C/120min e 120°C/20min. O efeito da concentração de vinhaça e do pH inicial (5, 6 e 7) e a produção de ácidos também foram avaliados. As comunidades microbianas foram analisadas por isolamento, PCR-DDGE e sequenciamento de alto desempenho dos genes 16S e 18S rRNA. O aumento da concentração de vinhaça afetou positivamente a produção de hidrogênio, sendo a produção máxima de hidrogênio (Hmax) (821,34 mL) e rendimento (4,75 mmol H_2 g^{-1}COD) obtida utilizando-se vinhaça não diluída a pH 6 e pré-tratamento do inóculo 90°C/10min (T1). Os ensaios T2 (105° C/120 min pH 5) e T3 (121°/20 min pH 6) produziram 687,58 mL e 697,68 mL respectivamente. Nos experimentos com a melhor produção de H_2, a rota metabólica butírica foi favorecida. O maior número de cópias do gene da Fe-hidrogenase está correlacionado com a amostra da maior produção de H_2. Isolamento e PCR-DGGE mostraram a presença de microrganismos anaeróbios facultativos (*Bacillus* e *Enterobacter*) e *Clostridium* nas comunidades microbianas. As análises do sequenciamento de alto desempenho revelaram alta prevalência do gênero *Clostridium* nas amostras pré-tratadas, com predominância de Firmicutes, Outros gêneros, como *Bacillus*, *Syntrophomonas*, *Geobacter*, *Syntrophus* e *Sulfurimonas*, também foram detectados no ensaio de maior produção de H_2 (T1). A análise do gene 18S rRNA para o ensaio T1, mostrou a prevalência de *Candida* (47%) e também a presença de Agaricomycetes, Pezizomycotina e *Aspergillus*. Os resultados deste estudo mostram a

prevalência de Clostridiaceae nos 3 ensaios (T1, T2 e T3) e, diminuição da diversidade microbiana com o aumento das temperaturas dos pré-tratamentos. O ensaio T1 (T90 pH 6) de melhor desempenho favoreceu os microrganismos descritos como produtores de H_2. Os resultados deste estudo mostram a complexidade dos consórcios microbianos com o envolvimento de bactérias e fungos, sugerindo a necessidade de interações entre estes na produção de hidrogênio e ácidos graxos voláteis a partir degradação da vinhaça *in natura*, tornando o processo ecologicamente sustentável.

Palavras-chave: comunidade microbiana; pré-tratamento; hidrogênio; vinhaça; isolamento, sequenciamento de alto desempenho.

ABSTRACT

The use of hydrogen as a fuel is considered a promising alternative for the next decades, as a clean energy source and produce only water as a product of its combustion. The biological production of hydrogen can be carried out using various agroindustrial residues. Vinasse is a residue of ethanol production generated in the proportion of 12 to 15 liters per liter of ethanol produced. Currently, vinasse is mainly used as fertilizer, in the sugarcane crop itself, however, the environmental agencies are limiting the amount of vinasse that can be applied to the soil due to its high pollutant. The process of converting vinasse into hydrogen can be carried out using pure cultures or microbial consortia. The use of consortia may be more advantageous because they are easier to handle and does not require sterile media. However, some microorganisms may be contained in the consortia H_2 consumers, which makes it necessary to subject the inoculum to a pretreatment to suppress the activity of these bacteria. Thus, the objective of this work was to evaluate three methods of thermal pretreatment of the inoculum for the production of hydrogen and volatile fatty acids at different initial values of pH using vinasse as substrate and to compare the composition of the microbial community. The tests for H_2 production were performed using vinasse at 37 °C with the following pretreatments of the inoculum: 90 °C / 10min; 105 °C / 120min and 120 °C / 20min. The effect of vinasse concentration and initial pH (5, 6 and 7) and acid production were also evaluated. The microbial communities were analyzed by isolation, PCR-DDGE and high-performance sequencing of the 16S and 18S rRNA genes. The increase of the vinasse concentration positively affected the hydrogen production, with the maximum production of hydrogen (Hmax) (821.34 mL) and yield (4.75 mmol $H2g^{-1}COD$) obtained using undiluted vinasse at pH 6 and pretreatment of the inoculum 90 °C / 10min (T1). The assays T2 (105°C / 120 min pH 5) and T3 (121°C / 20 min pH 6) produced 687.58 mL and 697.68 mL, respectively. In the experiments with the best production of H_2, the butyric metabolic route was favored. The highest number of copies of the Fe-hydrogenase gene is correlated with the sample of higher H_2 production. Isolation and DGGE showed the presence of facultative anaerobic microorganisms (*Bacillus* and *Enterobacter*) and Clostridium in the microbial communities. High-performance sequencing analyzes revealed a high prevalence of the genus *Clostridium* in the pretreated samples, with a predominance of Firmicutes. Other genus, such as *Bacillus*, *Syntrophomonas*, *Geobacte*r, *Syntrophus* and *Sulfurimonas*, were also detected in the higher H_2 production assay (T1). Analysis of the 18S rRNA gene for the T1 showed the prevalence of *Candida* (47%) and also the presence of Agaricomycetes, Pezizomycotina and *Aspergillus*. The results of this study show the prevalence of Clostridiaceae in the 3 trials (T1, T2 and T3) and decrease of the microbial diversity with the increase of the temperatures of the pretreatments. The assay T1 (T90 pH 6) improved performance favored microorganisms described as producing H2. The results of this study show the complexity of

microbial consortia with the involvement of bacteria and fungi, suggesting the need for interactions between them in the production of hydrogen and volatile fatty acids from degradation of fresh vinasse, making the process ecologically friendly.

Keywords: microbial community; inocula pretreatment; hydrogen; vinasse; microrganisms isolation, high-performance sequencing.

1. INTRODUÇÃO

Atualmente, devido às questões ambientais provocadas pelas atividades antropogênicas, que estão modificando o clima do planeta, está havendo um esforço dos meios científicos e econômicos para diversificar a matriz energética, composta em sua maioria por combustíveis fósseis, que quando em combustão liberam gases como o dióxido de carbono, responsável pelo efeito estufa e outros gases poluentes. Na busca por fontes de energia mais sustentáveis, entre outras alternativas energéticas, o hidrogênio apresenta-se como combustível promissor, devido a sua grande eficiência energética e por produzir apenas água, como produto da combustão.

A produção de hidrogênio por processos fermentativos tem recebido muita atenção nos últimos anos, principalmente devido a pequena demanda de energia. Estes processos fermentativos geralmente também possuem como produtos finais compostos que podem ser aproveitados como insumos químicos e ainda tem o potencial de utilizar diversos tipos de resíduos agroindustriais como substrato, reduzindo a quantidade de descarte dos mesmos no meio ambiente.

Entre os principais coprodutos derivados das rotas metabólicas microbianas estão os ácidos graxos voláteis e os álcoois, os quais também possuem valor agregado, podendo ser utilizados em várias aplicações na indústria têxtil, alimentícia, farmacêutica e plásticos. Portanto a produção fermentativa de hidrogênio pode ser considerada sustentável, principalmente pelo fato de utilizar fontes renováveis e devido a geração dos diversos tipos de ácidos carboxílicos.

Os processos biológicos para a produção de gás hidrogênio e outros coprodutos podem ser dependentes de energia luminosa, como a fotofermentação utilizando bactérias fototróficas ou por via fermentativa, utilizando bactérias anaeróbias facultativas e/ou anaeróbias estritas. Entre os principais microrganismos descritos como produtores de hidrogênio via fermentação mesofílica, estão os pertencentes aos gêneros *Clostridium*, *Klebsiella*, *Enterobacter* e *Bacillus*.

O processo fermentativo pode ser realizado por culturas puras ou por consórcios microbianos. Estes últimos, quando em aplicações em grande escala, podem ser mais vantajosos, pois não há a necessidade de esterilização do meio de cultivo e se pode utilizar uma variedade de resíduos como substrato. Outro aspecto importante da utilização de consórcios é que podem ser utilizadas fontes naturais de inóculos, como por exemplo: amostras de solo, lodo de digestão anaeróbia, águas residuárias de tratamento de esgoto, aterro sanitário doméstico, entre outros. No entanto, a produção de hidrogênio por consórcios microbianos requer métodos de pré-tratamento do inóculo para suprimir a atividade de microrganismos consumidores de hidrogênio, como bactérias homoacetogênicas e arqueias metanogênicas. Os métodos de pré-tratamento térmico têm sido amplamente descritos na literatura e variam de 65 °C a 121 °C.

O uso de múltiplos microrganismos requerem metodologias que identifiquem grupos microbianos que convivem associados nos processos fermentativos. Para isso, a técnica de

eletroforese em gel de gradiente desnaturante (DGGE), associada a PCR (reação em cadeia de polimerase), vem sendo amplamente empregada para a identificação de consórcios microbianos, inclusive os envolvidos na produção de hidrogênio e demais produtos. Devido ao seu gradiente desnaturante, com o DGGE é possível separar diferentes sequências de DNA dos principais microrganismos dominantes na comunidade microbiana. Também, nos últimos anos a identificação dos consórcios microbianos por sequenciamento de alto desempenho, tem se tornado uma importante ferramenta para os estudos de fermentação, ao apresentar a capacidade de identificar até 98% da população microbiana durante as etapas do processo, permitindo uma análise altamente representativa dos microrganismos atuantes na produção de hidrogênio e ácidos graxos voláteis. Aliado a isto, a PCR em tempo real para a quantificação do número de cópias de genes ligados a produção de hidrogênio (hidrogenases) de bactérias presentes nos consórcios, contribui para um melhor entendimento da dinâmica microbiana, servindo de complemento para monitorar diferentes fases do processo fermentativo.

Entre os substratos mais utilizados nos estudos de bioprodução de hidrogênio, destacam-se a glicose, sacarose, amido e a celulose. Todavia, para que a produção de hidrogênio seja um processo exequível e vantajoso em grande escala, é importante a utilização de substratos de baixo custo, como os resíduos agroindustriais, o que torna o processo ainda ecologicamente correto.

O Brasil está entre os maiores produtores mundiais de etanol e, tem se destacado, desde 1970, pelo programa de produção de etanol a partir da cana-de-açúcar, devendo se expandir também no futuro para o etanol de milho. Entretanto, para cada litro de etanol produzido, ocorre a geração de 13 a 15 litros de vinhaça, que é o principal resíduo líquido desta produção. A vinhaça é um líquido marrom escuro que possui elevada Demanda Química de Oxigênio (DQO), entre 17 e 50 g/L, e alta concentração de nitrogênio, fósforo, potássio, cálcio e magnésio sendo este resíduo normalmente utilizado como fertilizante no próprio cultivo da cana-de-açúcar e para a obtenção de biogás. Entretanto, face ao grande volume produzido a cada safra de cana-de-açúcar, impõe-se a necessidade de que sejam buscadas outras aplicações para este material.

Diante disso, o objetivo deste trabalho foi avaliar três métodos de pré-tratamento térmico de inóculo microbiano de lodo granular, para a produção de hidrogênio e ácidos graxos voláteis. As fermentações foram conduzidas a diferentes valores iniciais de pH, a partir de vinhaça, sendo também determinada e comparada a composição da comunidade microbiana antes e após os processos fermentativos e, a quantificação do gene da Fe-hidrogenase destes ensaios.

2. OBJETIVOS

2.1 Objetivo geral

Caracterizar e identificar consórcios microbianos presentes em inóculos com capacidade de produção de hidrogênio e outros coprodutos a partir de vinhaça de cana-de-açúcar, após pré-tratamento térmico do inóculo.

2.2 Objetivos específicos

- Caracterizar a composição química da vinhaça.
- Investigar a capacidade de produção de hidrogênio de consórcios microbianos em meio de cultivo contendo vinhaça como única fonte de nutrientes.
- Verificar a influência de diferentes concentrações de vinhaça na produção de hidrogênio.
- Avaliar métodos de pré-tratamento térmico do inóculo proveniente de lodo granular na produção de hidrogênio em meio de cultivo contendo vinhaça.
- Analisar a influência de diferentes valores de pH inicial na produção de hidrogênio em meio de cultivo contendo vinhaça.
- Avaliar a produção de ácidos graxos voláteis nos ensaios de produção de hidrogênio em meio de cultivo contendo vinhaça.
- Quantificar o número de cópias dos genes da Fe-hidrogenase por RT-PCR em inóculos com melhor produção de hidrogênio.
- Identificar os microrganismos constituintes dos consórcios microbianos por PCR-DGGE nos ensaios fermentativos de produção de hidrogênio em meio de cultivo contendo vinhaça.
- Caracterizar os consórcios microbianos com maiores capacidades de produção de hidrogênio por sequenciamento de alto desempenho dos genes 16S e 18S rRNA.
- Isolar os microrganismos anaeróbios e anaeróbios facultativos dos ensaios de melhor produção de hidrogênio.
- Avaliar a produção de hidrogênio dos microrganismos isoladamente em ensaio em meio de cultivo contendo vinhaça.

3. REVISÃO BIBLIOGRÁFICA

3.1 Hidrogênio como alternativa energética

O crescimento econômico e a rápida urbanização exigem cada vez mais energia para abastecer esta demanda mundial. O consumo primário de energia aumentou de cerca de 9.400 milhões de toneladas de petróleo em 2000 para mais de 13.000 milhões toneladas de petróleo em 2015 (Wang &Yin, 2017).

Atualmente, 87% das necessidades mundiais de energia são fornecidas por fontes fósseis, sendo o petróleo (33%) usado principalmente para transporte e na petroquímica, enquanto que o carvão (30%) é um dos pilares da produção de eletricidade para uso em indústrias e o gás natural (24%) cujo emprego vem se expandindo em todos os setores da sociedade (Liu, 2008; Yildiz, 2018).

No entanto, as reservas primárias de energia (combustíveis fósseis) dificilmente podem atender esta rápida e crescente demanda, o que força a busca de fontes alternativas de energia, menos poluentes, que tendem a aumentar de 3% (hoje) para 9% nos próximos 20 anos (Wang &Yin, 2017).

Entre as fontes de energias alternativas, encontram-se o hidrogênio (H_2), que é uma forma limpa e renovável de energia, sendo considerado um combustível promissor para o futuro, devido a possuir a maior quantidade de energia por unidade de massa em relação aos outros combustíveis e produzir apenas vapor d'água como produto resultante de sua combustão, não ocorrendo à formação de CO, CO_2 e hidrocarbonetos, que contribuem para o efeito estufa (Lay *et al*., 1999; Cheong & Hansen, 2006; Liu, 2008).

Precisamente, a combustão, altamente energética, do hidrogênio é de 122 kJ/g, sendo 50% mais eficiente do que a gasolina em automóveis (Liu, 2008), e seu manuseio é mais fácil e seguro do que o gás natural (Das *et al*., 2008). Apesar de seu alto potencial como combustível, o destino atual do hidrogênio está limitado somente a poucos processos industriais (Levin & Azbar, 2012). O interesse pelo hidrogênio tem aumentado devido às diversas possibilidades de aplicações que incluem o uso como combustível, em transportes e unidades de energia, na indústria química (por exemplo, refino de petróleo, produção de fertilizantes) e ainda oferece a possibilidade de gerar energia elétrica, química e energia térmica (Fonseca et al., 2019). O Canadá é o maior produtor e usuário per capita de hidrogênio, com 3,4 milhões de toneladas de hidrogênio produzido com 2,97 milhões de toneladas de consumo por ano (Levin & Azbar, 2012).

Acredita-se que para o futuro, o hidrogênio será essencial para a sustentabilidade energética e ambiental. Entretanto, ainda não está disponível em quantidade suficiente e sua produção ainda não é economicamente viável, fazendo a sua disponibilidade continuar a ser um desafio (Tansksale *et al.*, 2010). O maior obstáculo à utilização do hidrogênio como combustível é o seu custo de produção. Os atuais métodos de produção de hidrogênio são caros, demandam bastante energia, e são potencialmente nocivos para o meio ambiente (Levin & Azbar, 2012). Cerca de 96% do hidrogênio produzido no mundo é proveniente de uma fonte fóssil de energia (Ewan & Allen, 2005; Guo *et al*., 2012).

O H_2 pode ser produzido por uma série de processos, incluindo eletrólise da água, combustíveis fósseis e por processos biológicos. Existem diversos métodos que podem gerar hidrogênio biologicamente, como por meio de fotossíntese, biofotólise e foto-fermentação, os quais são dependentes de energia luminosa (Matsunaga *et al*., 2000; Kapdan & Kargi, 2006), ou via fermentação utilizando diversos tipos de carboidratos (Fang *et al*., 2002).

Na fermentação (*dark fermentation*) os carboidratos são convertidos em H_2, CO_2 e ácidos orgânicos, e é considerada uma alternativa promissora diante de processos dependentes de energia luminosa, em particular, quando se utiliza a biomassa de resíduos como matéria-prima (Levin & Azbar, 2012). Uma vez que a fermentação não requer um fornecimento constante de luz, este processo pode ser executado usando continuamente sistemas mais simples que podem ser utilizados comercialmente. Além disso, as taxas de produção de hidrogênio são muito mais elevadas utilizando fermentações, quando comparado com os sistemas à base de fotossíntese, reduzindo assim os custos do processo (Levin & Azbar, 2012; Levin *et al*., 2004).

A produção de H_2 via fermentação é o resultado da transferência de elétrons (e^-) para prótons (H^+) e, com o requisito mínimo de energia disponível nos compostos orgânicos durante o metabolismo anaeróbio (Dahiya *et al.*, 2015) sendo, um processo para a reoxidação das coenzimas (NADH) e manutenção de energia celular, pela produção de energia como ATP.

Portanto, a geração de biohidrogênio oferece um potencial de produção de energia renovável a partir de matérias-primas mais econômicas, resíduos industriais e agrícolas, tais como celulose (Levin *et al.*, 2006; Ratti *et al.*, 2015), glicerol (Suhaimi et al., 2012; Amaral *et al.*, 2009), vinhaça (Fernandes *et al.*, 2010; Lazaro *et al*., 2014), soro de queijo (Rosa *et al.*, 2014), amido (Chen et al., 2007), entre outros.

Embora o hidrogênio seja considerado uma alternativa apropriada em termos de tecnologias de energia limpa, sua baixa densidade em condições ambientais torna seu armazenamento e transporte um verdadeiro desafio (Verdinelli et al., 2019). Conforme relatam Levin & Azbar (2012), existem ainda muitos desafios científicos e de engenharia que devem ser cumpridos pelos atuais e futuros pesquisadores da área de biohidrogênio, para que esta tecnologia seja economicamente viável. Não é suficiente simplesmente demonstrar que um microrganismo pode produzir hidrogênio a partir de um ou outro substrato. As pesquisas de

biohidrogênio devem fornecer uma compreensão mais profunda de como o H_2 é produzido pelos microrganismos sob diferentes circunstâncias, e fornecer hipóteses testáveis de como aumentar esta produção.

3.2 Metabólitos solúveis presentes no processo de produção de hidrogênio

Os processos fermentativos para a produção de hidrogênio também estão associados com a geração de produtos secundários, como ácidos orgânicos de baixo peso molecular (ácidos graxos voláteis), especialmente acetato e butirato, que são os principais produtos orgânicos da fermentação (Lazaro *et al.,* 2015). Mas também, propionato, ácido lático, etanol, assim como 1,3-propanodiol, butanol, 2,3-butanodiol são produzidos em menores quantidades (Uyar *et al.*, 2009). Estes produtos serão formados de acordo com o microrganismo, suprimento ou não de oxigênio e tipo de substrato utilizado. A formação destes metabólitos solúveis está inter-relacionada com a formação de H_2 e o balanço de NADH (Zhang *et al*., 2015).

Os ácidos graxos voláteis, incluindo o ácido acético, propiônico e butírico podem se tornar valiosos substratos para produtos finais mais elaborados (Fu & Holtzapple, 2010), sendo utilizados para diversas finalidades e são gerados principalmente pela indústria petroquímica, por processos não renováveis e poluentes (Huang *et al.,* 2002).

O ácido acético (CH_3COOH) é um importante composto na indústria química e alimentícia, é o componente principal do vinagre e também é reconhecido por ser um composto antimicrobiano eficaz que impede o crescimento de organismos patogênicos e deteriorantes, em alimentos fermentados (Gullo, *et al*., 2014). Também pode ser empregado para produzir acetato de cálcio-magnésio, o qual é utilizado como um descongelante, utilizado em pistas de aeroportos, pontes e estradas (Huang *et al.,* 2002; Huo *et al.,* 2015).

O ácido butírico ($CH_3CH_2CH_2COOH$) é um composto químico com muitas aplicações na indústria alimentícia, farmacêutica e química. Ele pode ser utilizado como ácido para melhorar sabores amanteigados em alimentos ou em forma de ésteres para aumentar a fragrância de frutas e, ainda como compostos aromáticos para a produção de perfumes (Playne, 1985; Jiang *et al*., 2009).

Enquanto que o ácido propiônico (CH_3CH_2COOH) é amplamente utilizado como antifúngico, como conservantes em alimentos e como um intermediário na síntese de herbicidas, acetato de celulose, propionato, plásticos, solventes e produtos farmacêuticos (Kośmider *et al*., 2010; Coral *et al*., 2008).

Entre os álcoois gerados nos processos fermentativos, estão o butanol, etanol e o 2,3-butanodiol. Dos biocombustíveis, o butanol ($CH_3CH_2CH_2CH_2OH$) é particularmente valioso porque possui muitas propriedades físicas favoráveis, tendo o teor de energia próximo ao da gasolina (Raganati *et al.,* 2015). Enquanto que o 2,3-butanodiol ($CH_3CH(OH)CH_2CH_2OH$) pode

ser facilmente convertido em metil etil cetona, um aditivo de combustível líquido e, em 1,3-butadieno que é polimerizado e usado na fabricação de fibras sintéticas e de borracha (Kim *et al*., 2013). E, o etanol (CH_3CH_2OH), utilizado isoladamente ou suplementado com a gasolina como um combustível, para o transporte e também pode ser utilizado como um substrato para a produção de biodiesel (Han *et al*., 2011).

Atualmente, tem havido um interesse crescente na produção destes compostos orgânicos por processos fermentativos (Atasoy et al., 2018), devido as preocupações sobre o fornecimento e o eventual esgotamento mundial das reservas de petróleo e, também ao acordo global de alguns países que visa para a redução de emissões de gases de efeito estufa até 2050 (Atasoy et al., 2018).

Vários processos fermentativos vêm sendo estudados para a bioprodução destes compostos a partir de glicose (Infantes *et al*., 2011), celulose (Sarah et al. 2012), xilose (Liu & Yang, 2006) e sacarose (Vandák et al., 1995). Contudo, para que o processo de fermentação seja economicamente viável e se torne uma alternativa atraente, devem-se utilizar subprodutos industriais de baixo valor como fontes de carbono, como por exemplo: resíduos sólidos urbanos (Sans *et al*., 1995), resíduos alimentares (Lim *et al*., 2008), soro de queijo (Bengtsson *et al.*, 2008), diversos tipos de efluentes industriais (Lee *et al.*, 2014), glicerol residual (Tan *et al*., 2013), vinhaça de cana de açúcar (Lazaro et al., 2014), entre outros (Lee *et al.*, 2014).

3.3 Etapas de um processo de fermentação e a produção de hidrogênio

A fermentação consiste em um processo biológico no qual uma cultura pura ou consórcios de diferentes tipos de microrganismos promovem a transformação de compostos orgânicos complexos (carboidratos, proteínas e lipídios) em produtos mais simples, tais como ácidos orgânicos voláteis (ácidos acético, propiônico, isobutírico e butírico), álcoois (etanol, butanol), H_2, CO_2 e CH_4, sem a participação de oxigênio (Sá *et al*., 2014).

O processo de fermentação é desenvolvido principalmente pelas seguintes etapas: hidrólise, acidogênese, acetogênese e metanogênese (Figura 1). Na primeira fase do processo fermentativo, bactérias hidrolíticas produzem enzimas extracelulares que promovem a degradação dos materiais particulados complexos em materiais dissolvidos mais simples, os quais são permeáveis às membranas celulares das bactérias fermentativas (Sá *et al*., 2014). Na fase acidogênica, os produtos solúveis oriundos da etapa anterior são metabolizados no interior das células das bactérias, sendo convertidos em compostos mais simples. Os compostos produzidos incluem ácidos orgânicos voláteis, álcoois, CO_2, H_2, além de novas células bacterianas. As bactérias acetogênicas são responsáveis pela oxidação dos produtos gerados na fase acidogênica em substrato apropriado (H_2 e ácido acético) para as arqueias metanogênicas. Nesta etapa do processo, o H_2 pode também ser convertido em ácido acético pelas bactérias

homoacetogênicas. Na última etapa do processo, as arqueias metanogênicas convertem o H_2 e o ácido acético em CH_4 e CO_2 (Sá *et al*., 2014).

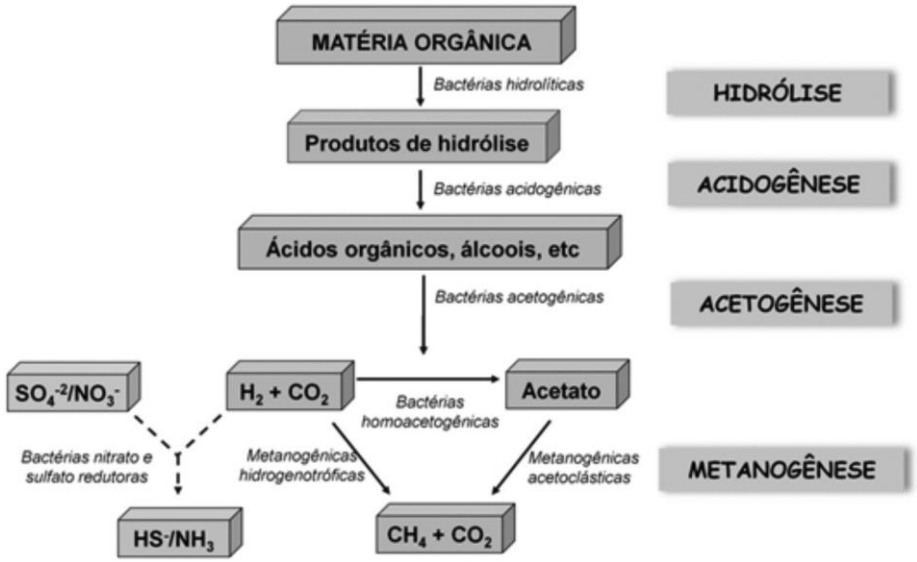

Figura 1: Representação esquemática das principais etapas do processo fermentativo anaeróbio (Sá *et al.*, 2014).

O processo biológico para a obtenção de hidrogênio pode ser realizado utilizando-se culturas puras ou por consórcios de bactérias (Amaral et al., 2009, Lay et al., 2010) anaeróbias e anaeróbias facultativas crescidas no escuro com substratos ricos em carboidratos. As reações de fermentações podem ocorrer em condições mesofílicas (25-40°C), termofílicas (40-65°C) ou hipertermofílicas (>80°C) (Levin *et al*., 2004). Os processos fermentativos produzem uma mistura de biogás contendo principalmente H_2 e CO_2, mas que também podem conter menores quantidades de CH_4, CO, e / ou sulfeto de hidrogênio (H_2S) (Levin *et al*., 2004).

Como a produção de H_2 é um processo anaeróbio, não há problemas relacionados à limitação por O_2 (Kotay & Das, 2008). As bactérias anaeróbias facultativas são menos "sensíveis à presença de oxigênio" e têm a capacidade de produzir hidrogênio em caso de esgotamento de O_2 no meio. Como consequência, as bactérias anaeróbias facultativas são, em geral, mais indicadas na condução de um processo de produção de H_2 por fermentação (Das & Veziroglu, 2008).

A bioprodução de hidrogênio depende de uma série de condições e parâmetros operacionais e físico-químicos que influenciam o processo, nomeadamente, o tipo de inóculo e de substrato, o tipo de reator, a temperatura, a presença e a concentração de nutrientes assim como o pH do meio (Xiao *et al*., 2010; Wang & Wan, 2009). Uma das vantagens da produção de hidrogênio por via biológica está relacionada à capacidade dos microrganismos de selecionarem o seu substrato, mesmo quando este se encontra numa mistura de outros compostos. Deste modo, minimiza-se a necessidade de purificação do substrato fundamental ao processo, o que se traduz num maior espectro de matérias-primas que poderiam ser utilizados e

uma menor necessidade de tratamentos químicos (Drapcho *et al.*, 2008). Além disso, o emprego de temperatura e pressão de operação similar às ambientais consistiria em uma vantagem relevante do processo microbiológico, tornando-se um processo com balanço energético favorável (Das *et al*., 2008).

3.3.1 Consórcios microbianos na produção de hidrogênio

A utilização de culturas mistas para processos em grande escala é considerada vantajosa devido ao processo ser facilitado pela utilização de meios não estéreis, reduzindo o custo global. A redução do custo pode ser ainda enfatizada quando empregadas culturas mistas a partir de fontes naturais, tais como solo, lodo de esgoto, excreta de animais ou resíduos industriais e domésticos (Sá *et al.,* 2014; Maintinguer *et al.,* 2015). No entanto, a maioria dos inóculos descritos são predominantemente obtidos a partir de países de clima temperado. Existem poucos estudos com a produção de hidrogênio usando inóculos de países tropicais, como o Brasil, onde as temperaturas são em torno de 25° C, ideais para o crescimento bacteriano (Maintinguer *et al*., 2015).

Os consórcios microbianos, não se baseiam em uma estirpe específica de microrganismo e por isso pode ser operado em condições não estéreis, sem risco significativo de contaminação (Lu *et al.*, 2011). Além disso, os consórcios são capazes de consumir um amplo espectro de substratos contendo diversos tipos de compostos químicos orgânicos (Rodriguez *et al.*, 2006).

Vários microrganismos anaeróbios presentes em consórcios produzem hidrogênio a partir de compostos orgânicos contidos em diferentes tipos de resíduos. Destacam-se, predominantemente nos consórcios, bactérias mesófilas anaeróbias estritas pertencentes ao gênero *Clostridium*, como *C. butyricum*, *C pasteurianum*, *C. paraputrificum* e *C. bifermentants* (Sivagurunathan *et al*., 2014). Estes microrganismos representam cerca de 65% do total das populações produtoras de H_2 estudadas, podendo ser facilmente encontrados em consórcios microbianos de lodos ativados e obtidos a partir de tratamento térmico. As espécies do gênero *Clostridium*, particularmente, produzem hidrogênio durante a fase exponencial de crescimento, dado que, ao atingir a fase estacionária, o metabolismo celular altera-se, passando da produção de hidrogênio para a produção de outros compostos (Chong *et al*., 2009; Yossan *et al.*, 2012).

Outros microrganismos anaeróbios facultativos produtores de hidrogênio, pertencentes à família Enterobacteriaceae, também estão presentes em consórcios microbianos. Estudos descrevem a presença de culturas de *Enterobacter cloacae* (Maintinguer *et al*., 2008), *Klebsiela pneumonae* (Rossi *et al.*, 2011). A presença de microrganismos do gênero *Bacillus* também tem sido encontrada em culturas mistas na produção de hidrogênio (Wang *et al.*, 2010; Motte *et al*., 2014).

Diversos trabalhos obtiveram rendimentos consideráveis na produção de hidrogênio a partir de inúmeros tipos de consórcios com diversas fontes de substratos (Tabela 1). Maintinguer et al, (2008), obtiveram um rendimento de 1,6 mol H_2/mol de sacarose utilizando como inóculo efluente de suinocultura. Enquanto que Rossi *et al.*, 2011, com a utilização de inóculo granular proveniente de uma estação de tratamento de soja, obtiveram uma produção de 34,19% mol de hidrogênio, com glicerol residual como substrato. Em trabalho do nosso grupo, Schiavenin et al., obtiveram um rendimento de 1.02 molH_2/mol glicerol residual em ensaios em batelada utilizando como inóculo lodo anaeróbio granular de indústria de óleo vegetal. Em outro trabalho, com consórcio termotolerante obtido de lodo anaeróbio Yossan et al, (2012), encontraram o potencial máximo de produção de hidrogênio de 702,52 mL/L de óleo de palma. Enquanto, Davila-Vazquez et al. (2009), com a utilização de consórcios proveniente de lodo anaeróbio granular e soro de queijo como substrato, obtiveram 2.8 mol H_2/mol lactose em ensaios em reator contínuo durante 65 dias. Lazaro et al. (2015), criaram um consórcio microbiano artifical composto por microrganismos fototrópicos (*Rhodobacter*, *Rhodospirillum*, *Rhodopseudomonas* e *Sulfurospirillum*) e obtiveram 41,5 mmol H_2/L e, uma remoção de DQO de 95%, utilizando acetato de sódio e butirato de sódio como fontes de carbono, em ensaios em batelada com constante iluminação. Lui et al. (2019) obtiveram uma produção máxima de hidrogênio de 1591 mL H_2 / L (equivalente a 1,015 mol H_2/mol de glicose), utilizando amido como substrato e consórcio proveniente de lodo ativado (Tabela 1).

Tabela 1: Produção de hidrogênio por consórcios microbianos

Inóculo	Substrato	Produção de H_2	Referência
Efluente de suinocultura	Sacarose	1,6 mol H_2/ mol	Maintinguer et al, (2008)
Lodo aneróbio granular	Soro de queijo	2,8 mol H_2/ mol	Davila-Vazquez et al. (2009)
Lodo granular	Glicerol residual	34,19% de H_2	Rossi *et al.*, 2011
Lodo aneróbico	Efluente de óleo de palma	702,52 mL / L	Yossan et al, (2012)
Consórcio artifical	Acetato de sódio e butirato de sódio	41,5 mmol H_2/L	Lazaro et al. (2015)
Lodo ativado	Amido	1591 mL H_2 / L	Liu et al. (2019)
Inóculo granular	Glicerol residual	1,02 mol H_2 / mol	Schiavenin et al. (em preparação)

Contudo, o H_2 produzido por estes consórcios, quando extraído do sistema pode ser utilizado como um combustível, mas quando retido no sistema ele age como um doador de elétrons para produzir tanto ácido acético (homoacetogênese) quanto metano (metanogênese hidrogenotrófica) (Noori & Saady, 2013). No processo, bactérias acidogênicas facilitam a formação de ácido acético, propiônico, butírico, valérico e os ácidos graxos de cadeia superior (C3 ou mais), são posteriormente oxidados para ácido acético através da ação de bactérias

sintróficas (bactérias acetogênicas produtoras de H_2). O ácido acético também pode ser produzido por bactérias homoacetogênicas utilizando H_2 e CO_2. O hidrogênio produzido pode vir a ser consumido pelas bactérias consumidoras de hidrogênio.

Todavia, este consumo de H_2 no sistema consiste em uma desvantagem, pois induz a uma queda do rendimento final da produção. Uma das formas de contornar esse efeito seria a aplicação de métodos de pré-tratamento do inóculo, como o tratamento térmico (90-110°C) para suprimir a atividade de microrganismos consumidores de hidrogênio (arqueias metanogênicas), selecionando os formadores de endósporos, como *Clostridium*, *Bacillus e Thermoanaerobacterium*. Contudo, outros microrganismos formadores de endósporos, tais como *Acetobacterium*, *Propionibacterium*, *Sporolactobacillus* (produtores de ácido lático e propiônico, respectivamente), podem consumir hidrogênio (Kraemer & Bagley, 2007). Também, outros métodos como ácidos ou bases, arejamento, congelação e descongelamento, clorofórmio ou iodopropano (Wang & Wan, 2008; Rossi *et al*., 2011), são também indicados para eliminar os microrganismos consumidores de hidrogênio.

3.4 Metabolismo microbiano

3.4.1 Vias metabólicas para produção de hidrogênio

Diversos tipos de carboidratos tais como a glicose, sacarose, amido ou celulose, podem ser utilizados como substratos para a produção biológica de hidrogênio por processos fermentativos.

Na via metabólica de microrganismos produtores de H_2 (Figura 2), ocorre a oxidação de carboidratos gerando elétrons, que são dissipados para a formação de H_2, mantendo o equilíbrio redox intracelular (Fonseca, 2016). O piruvato formado a partir da oxidação do carboidrato na via glicolítica é convertido em acetil-CoA e CO_2, com a participação da enzima piruvato ferredoxina oxidorredutase (PFOR). A ferredoxina que compõe esta enzima é re-oxidada pela transferência de elétrons aos íons H^+, através da enzima hidrogenase, formando o hidrogênio molecular (H_2), gerando 2 mol de H_2 por mol de glicose. Durante a glicólise, também ocorre a re-oxidação do NADH, cujos elétrons podem ser transferidos à ferredoxina (PFOR), pela enzima NADH-ferrodoxina oxidoredutase (NFOR) e posteriormente aos íons H^+, gerando mais 2 mol de H_2 (Sinha & Pandey, 2011; Fonseca, 2016).

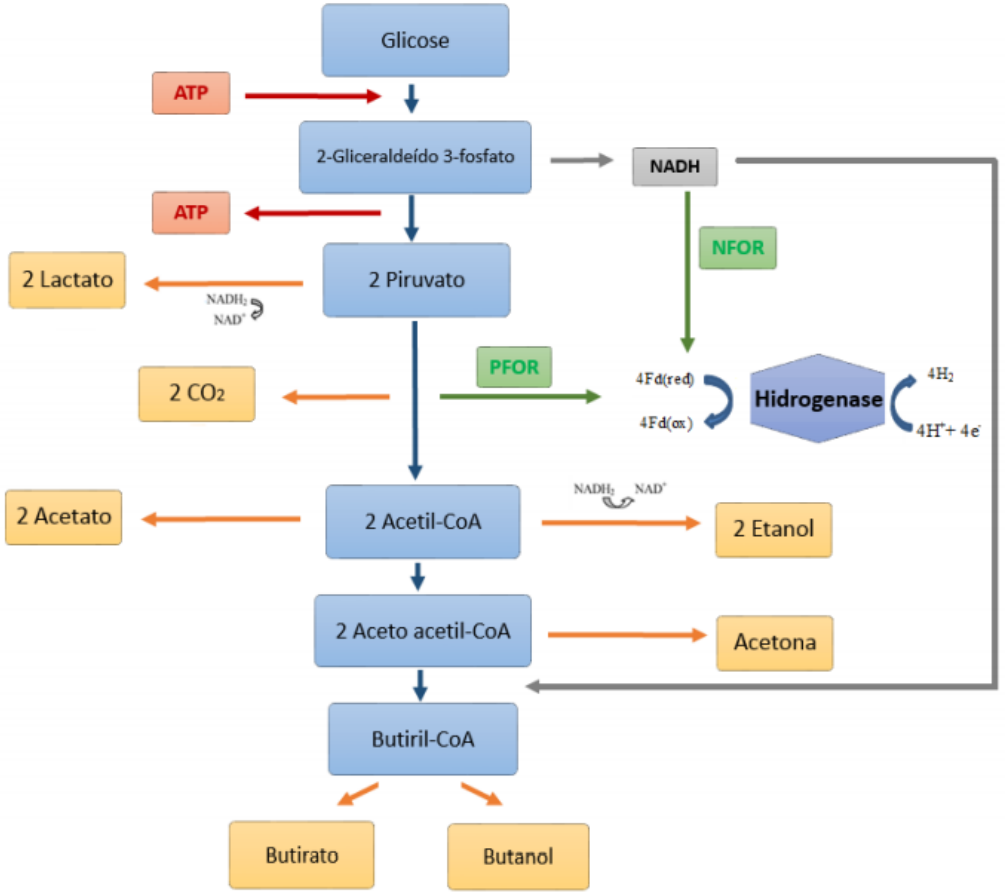

Figura 2: Via metabólica para conversão de glicose em H_2, CO_2, ácidos orgânicos e solventes por microrganismos do gênero *Clostridium* sp. (Fonseca, 2016).

A degradação metabólica dos carboidratos durante a glicólise produz também butirato, acetato, lactato, etanol, acetona, butanol como produtos finais da fermentação microbiana (Hallenbeck, 2012; Fonseca, 2016). Quando o ácido acético é o produto final, a conversão máxima teórica de 4 mol de H_2 por mol de glicose é obtida (equação 1). Porém, quando o ácido butírico é o produto final, ocorre a conversão de 2 mol de H_2 por mol de glicose (equação 2) (Levin *et al*., 2004), pois a formação dos co-produtos lactato, etanol, butanol ou ácido butírico consome NADH, reduzindo o rendimento global de hidrogênio.

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

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_2CH_2CH_2COOH + 2H_2 + 2CO_2 (2)$$

Já para a degradação metabólica de sacarose ocorre primeiramente o consumo da sacarose e geração de ácido acético, seguido pelo consumo da sacarose e geração de ácido butírico, como descrito nas equações 3 e 4 (Maintinguer *et al.*, 2008).

$$CH_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2 \quad (3)$$

$$C_{12}H_{22}O_{11} + H_2 \rightarrow 2CH_3CH_2CH_2COOH + 4CO_2 + 4H_2 \quad (4)$$

A conversão máxima teórica de sacarose para produção de hidrogênio é equivalente a 8 mol de hidrogênio por mol de sacarose. Quando a sacarose é convertida para butirato, ocorre a geração de 4 mol de hidrogênio por mol de sacarose consumida. A maior produção teórica de H_2 está associada com a produção de acetato como o produto final da fermentação. Por outro lado, em termos práticos, a maior produção de hidrogênio está relacionada com a produção de acetato e butirato como produtos da fermentação e, a baixa produção de hidrogênio está associada à produção de propionato, álcoois e ácido lático (Levin *et al*., 2004; Maintinguer *et al*., 2008).

O rendimento de hidrogênio é altamente dependente de diversas condições do processo, tais como pH, tempo de detenção hidráulica e a pressão parcial do reator, que afetarão o equilíbrio metabólico microbiano. Assim, os produtos finais da fermentação produzidos por uma bactéria dependem das condições ambientais em que ela cresce. A formação dos produtos reduzidos finais da fermentação como o etanol, butanol e lactato, reduz o rendimento de hidrogênio no processo. Para maximizar este rendimento, o metabolismo deve ser dirigido para longe dos álcoois (etanol, butanol) e ácidos reduzidos (lactato) para ácidos graxos voláteis (Levin *et al*., 2004). De acordo com Dabrock *et al.* (1992) o microrganismo *C. pasteurianum* que é um clássico produtor de H_2 e ácidos graxos voláteis, pode ter seu metabolismo inibido para a produção de H_2 e de ácidos graxos com elevadas concentrações de CO_2, que inibe a ação das hidrogenases, e desvia a rota metabólica para a produção de butanol, etanol e lactato. Também, o acúmulo de ácidos orgânicos no meio líquido, pode levar ao aumento da permeabilidade da membrana celular das bactérias, provocando a queda do pH interno celular, promovendo uma possível desnaturação da enzima Fe-hidrogenase, responsável pela produção de hidrogênio (Zhao *et al.*, 2010).

3.4.2 Hidrogenase

A capacidade de certos microrganismos em produzir o hidrogênio molecular foi descoberta no final do século 19 e mais tarde identificada por ser catalisada por uma enzima chamada hidrogenase. Desde então, têm sido observado e caracterizado hidrogenases em vários microrganismos, incluindo algumas algas, tricomonas, ciliados anaeróbios, fungos e bactérias.

Esta enzima catalisa a reação química (equação 5), com a formação redutiva e reversível de hidrogênio a partir de prótons e elétrons (Tamagnini *et al*., 2002), principalmente em microrganismos do gênero *Clostridium* (Sá et al., 2011).

$$2H^+ + 2e^- \leftrightarrow H_2 \quad (5)$$

As estruturas das hidrogenases são muito complexas e são classificadas de acordo com os metais presentes em seus sítios ativos: Fe-Fe, Ni-Fe e Fe-hidrogenase (contém somente um sítio ativo de Ferro) (Figura 3). A produção de hidrogênio por microrganismos que exercem fermentação é realizada tanto pela Ni-Fe hidrogenase quanto pela Fe-Fe hidrogenase (Hallenbeck, 2012). Praticamente, todas as classes de bactérias contém Ni-Fe-hidrogenase (Tamagnini *et al*., 2002).

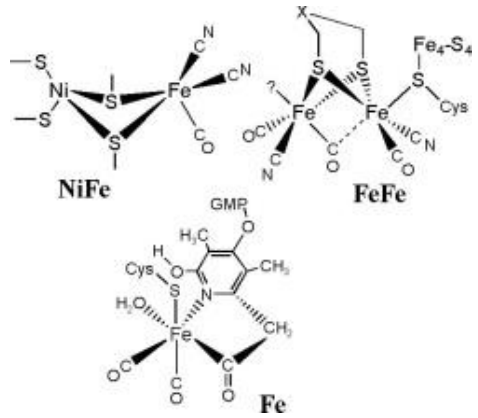

Figura 3: Estruturas dos sítios ativos das enzimas hidrogenases (Hallenbeck, 2012).

A enzima Ni-Fe-hidrogenase ocorre apenas em bactérias e Arqueas, enquanto que Fe-Fe- hidrogenase ocorre em bactérias e eucariotos (Hallenbeck, 2012). A Fe-Fe-hidrogenase normalmente ocorre em microrganismos produtores de hidrogênio como a classe Clostridia. Enquanto que a Ni-Fe-hidrogenase é frequentemente encontrada em microrganismos consumidores de hidrogênio, tais como as arqueobactérias metanogênicas, responsável pela conversão de H_2 em CH_4 (Sá *et al*., 2011).

Nos últimos anos, diversos estudos têm revelado um grande número de genes envolvidos na expressão e funcionamento da Ni-Fe-hidrogenase (Vignais & Colbeau, 2004; Hallenbeck, 2012). Alguns organismos possuem a capacidade genética para expressar múltiplas Ni-Fe-hidrogenases, como por exemplo, *Escherichia coli*, cujo genoma codifica quatro diferentes Ni-Fe-hidrogenases. As hidrogenases de espécies produtoras de hidrogênio, como os *Clostridium* já foram sequenciadas e caracterizadas, incluindo *C. pasteurianum* (Meyer &

Gagnon, 1991; Fang *et al.*, 2006), *C. acetobutylicum* (Santangelo *et al.*, 1995; Fang *et al*., 2006); e C. *paraputrificum* (Morimoto *et al.*, 2005; Fang *et al*., 2006).

Recentemente, o nível da expressão do gene hidrogenase (*hyd*) está sendo usado como indicador da produção de hidrogênio em diversos sistemas de produção (Sá *et al.*, 2011; Chang *et al.*, 2008; Perna *et al.*, 2013; Castelló et al., 2018).

3.5 Identificação dos microrganismos

A fim de determinar a composição da comunidade dos microrganismos que participam efetivamente da fermentação que resulta na produção de H_2 e, também para avaliar mudanças na estrutura das comunidades mistas, após a aplicação de certos métodos de pré-tratamento de inóculos (Wang & Wan, 2009; Kim *et al*., 2006), é imprescindível a realização de técnicas de identificação microbiana. As técnicas de microbiologia clássica, microscopia, testes bioquímicos e isolamento em diferentes meios de cultura enriquecidos em aerobiose e anaerobiose, são importantes para obter o microrganismo, conhecer as condições ideais de fermentação e projetar ensaios com os microrganismos isoladamente ou construir consórcios microbianos artificiais para melhorar a produção de hidrogênio (Fuentes et al., 2018).

Para a identificação dos microrganismos, diversos métodos de biologia molecular qualitativa e quantitativa são utilizados, tais como PCR (*Reação em Cadeia de Polimerase*), RT-PCR (*Reação em Cadeia de Polimerase em Tempo Real*), PCR-DGGE (PCR e *Eletroforese em Gel de Gradiente Desnaturante*) e sequenciamento de alto desempenho dos consórcios microbianos envolvidos no processo. As metodologias moleculares garantem não só a identificação em nível de gênero e espécie das linhagens presentes no reator, como também permite a quantificação da expressão gênica durante o processo fermentativo.

A reação de PCR consiste na replicação de determinada região do genoma de qualquer organismo, amplificando alguns milhões de vezes, o que facilita a análise genética e permite o desenvolvimento de diagnósticos muito mais sensíveis, através do uso da Taq-DNA polimerase e de primers (oligonucleotídeos) específicos que se ligam à cadeia de DNA orientando a síntese (Sena Oliveira *et al*., 2007). Esta técnica consiste em três fases: desnaturação da fita de DNA; hibridização (anelamento) de cada primer nas respectivas sequências complementares à região-alvo da amplificação; e extensão, onde a enzima Taq-DNA-polimerase se posiciona junto aos primers e inicia a síntese da nova fita de DNA. Estas três etapas juntas correspondem a um ciclo da reação. Após o término de um ciclo (desnaturação, anelamento e extensão), todo o processo é repetido várias vezes (25 a 35 vezes), até que se obtenha grande quantidade do DNA a ser amplificado. A cada ciclo de amplificação, o número de cópias da sequência-alvo é duplicado e,

com a evolução dos ciclos da reação, ocorre o aumento exponencial do número dessas sequências (Sena Oliveira *et al*., 2007).

O RT-PCR (PCR em Tempo Real) realiza a quantificação dos ácidos nucléicos (DNA e RNA) de maneira precisa e com maior reprodutibilidade, porque determina valores na fase exponencial da reação, baseando-se na emissão de sinais luminosos por compostos fluorescentes (SYBR Green ou TaqMan). Estes fluoróforos apresentam a propriedade de unirem-se a fita de DNA, resultando no aumento da fluorescência, gerando um sinal que aumenta na proporção direta da quantidade de produto de PCR. Sendo assim, os valores da fluorescência são gravados durante cada ciclo e representam a quantidade de produto amplificado. Suas vantagens em relação ao PCR são: quantificação do material genético, maior precisão, reprodutibilidade, velocidade da análise e menor risco de contaminação (Novais & Alves, 2004). Para quantificar o conteúdo do DNA de uma amostra é necessário construir uma curva de calibração, utilizando diluições seriadas de DNA que contenha o gene a ser quantificado, em uma ampla faixa de concentração.

A técnica de DGGE vem sendo aplicada para estudar diversidade de diferentes grupos de microrganismos em diversos habitats (Muyzer *et al*., 1993; Mocali et al., 2015; Li et al., 2018). Esta técnica pode ser utilizada para analisar o perfil da diversidade de uma comunidade microbiana quantitativamente e qualitativamente. Estudos com amostras de lodos ativados mostram que o método de PCR-DGGE pode ser uma excelente ferramenta para caracterizar a estrutura da comunidade microbiana no nível de gênero e espécie e para monitorar as mudanças da comunidade ao longo do tempo de fermentação (Onuki *et al*., 2000).

A análise de DGGE permite a identificação de consórcios microbianos por meio das diferenças nucleotídicas dos fragmentos de DNA (com os mesmos tamanhos) dos diferentes microrganismos que compõe a comunidade. A separação desses fragmentos ocorre em gel de poliacrilamida de acordo com grau de desnaturação da dupla hélice de DNA, sob a ação de agentes desnaturantes tais como formamida e uréia (Maintiguer, 2009). Devido as diferenças nucleotídicas de cada sequência, a medida que cada uma atinge o seu ponto de desnaturação, elas param de migrar no gel (Muyzer *et al*., 1993), permitindo a sua separação por DGGE.

Nos últimos anos, o sequenciamento de alto desempenho vem ganhando cada vez mais espaço no meio científico, devido ao rápido progresso desta tecnologia e a capacidade de gerar informação sobre milhões de pares de bases em uma única corrida, além do baixo custo do sequenciamento.

Análises de consórcios microbianos por sequenciamento de alto desempenho (*metabarcoding*) apresentam-se como estratégias adequadas para caracterizar a composição e dinâmica de populações, pois permitem a identificação dos microrganismos ali presentes em diversos tipos de amostras, sem necessidade de cultivo. A genômica ambiental tem o potencial de responder questões básicas da diversidade genética, estrutura populacional e ecologia de

diversos grupos, inclusive no caso de microrganismos ainda desconhecidos, não cultivados ou que sofreram alteração pelo meio em que se encontram (Riesenfeld *et al.* 2004).

Nesta abordagem, a análise da região codificadora do gene ribossomal (16S rRNA para procariotos) da subunidade menor do ribossomo, tem se tornado um dos métodos mais eficientes para a identificação de organismos. O gene 16S ribossomal é o gene mais utilizado na identificação das bactérias. Os amplicons gerados por PCR são sequenciados e as sequências comparadas a bancos de dados de referência para identificar as unidades taxonômicas operacionais (OTUs). O número de leituras de sequência identificadas com as mesmas OTUs é calculado e uma estimativa quantitativa é gerada para cada OTU na amostra analisada (Ercolini, 2013).

As milhares de sequências geradas pelo sequenciamento são comparadas através de algoritmos para busca de banco de dados de sequências que buscam alinhamentos locais e globais (Edgar, 2010). A partir destas análises é possível comparar as sequências e realizar a descoberta de novos genes, funções e características de uma nova sequência (Seibel *et al.* 2000).

3.6 Vinhaça como substrato na produção de H_2

O Brasil tem se destacado desde a década de 1970 pelo seu programa de etanol a partir da cana-de-açúcar. A geração de vinhaça no processo de destilação de etanol de cana-de-açúcar no Brasil vem se ampliando de acordo com o crescimento da indústria do etanol. Na destilaria para cada litro de etanol recuperado na destilação, são gerados aproximadamente 12 a 15 litros de vinhaça (Springer & Goissis, 1988; Nogueira *et al.*, 2015). Em geral, as indústrias utilizam tanques de decantação onde ocorre a evaporação na parte líquida da vinhaça e decantação dos constituintes sólidos dissolvidos. No entanto, o armazenamento da vinhaça por longos períodos nessas áreas propicia a sua infiltração no solo, podendo ocorrer contaminação do lençol freático (Fraga *et al*., 1994).

A vinhaça é um líquido de cor marrom escura, com pH ácido (3,0-5,0), possui elevada matéria orgânica (30 a 40 g/L DBO) e um poder poluente de 100 vezes mais que o esgoto doméstico. A composição química da vinhaça depende das características do solo, da variedade da cana-de-açúcar, da estação do ano em que é feita a colheita e do processo industrial usado para a produção de etanol (Salomon & Lora, 2009; Lazaro *et al*., 2014).

Atualmente, tanto a matéria orgânica quanto os sais minerais contidos na vinhaça podem ser recuperados sob a forma de matéria-prima para outras aplicações, como ração animal ou material para construção civil, ou ainda para uso direto, como fertilizante, no próprio cultivo da cana-de-açúcar (Salomon & Lora, 2009; Moraes *et al*., 2014; Parsaee et al. 2019). Do ponto de vista econômico, esta última aplicação representa a solução mais simples e acessível para destinar este efluente. No entanto, mesmo que seja permitido por lei, não se pode afirmar com

segurança se esta ação não resulta em impactos ambientais, pois a regulamentação brasileira prescreve a aplicação de vinhaça de acordo ao seu teor de potássio, e só prevê impactos causados pela vinhaça no solo, água, e águas subterrâneas, negligenciando o conteúdo de matéria orgânica e impactos atmosféricos (Moraes *et al*., 2014). Contudo, o uso a longo prazo da vinhaça em terras produtivas pode causar desertificação e salinização do solo, causando diminuição da produtividade, maturação tardia e diminuição do teor de sacarose na cana-de-açúcar (Sydney *et al.*, 2014)

Assim, seu aproveitamento racional, além de representar uma reciclagem de recursos naturais com valor agregado, permite atender com muito mais eficiência aos requisitos da legislação de controle de poluição (Fraga *et al*., 1994). Como alternativa, a digestão anaeróbia para a produção de hidrogênio, metano e outros compostos de interesse econômico, pode ser aplicada para o tratamento da vinhaça, reduzindo seu teor de matéria orgânica, mantendo a maioria dos seus nutrientes, tais como potássio, azoto, fósforo e, que são ligeiramente removidos através da assimilação metabólica para a manutenção celular (Moraes *et al*., 2014).

A fim de reaproveitar a vinhaça de cana-de-açúcar, Sydney *et al*., (2014) verificaram a produção de hidrogênio e ácidos graxos voláteis com consórcios microbianos utilizando vinhaça suplementado com sacarose, melaço e caldo de cana, verificando um rendimento de 7,14 molH_2/ mol de sacarose em meio contendo vinhaça e caldo de cana. Fernandes *et al*. (2010) verificaram um rendimento 25 mmol H_2/g de DQO em cultivo consorciado contendo vinhaça de cana-de-açúcar, em comparação aos substratos glicerina e esgoto doméstico. Por sua vez, Lazaro *et al*. (2014) observaram produção de hidrogênio de 2,31 mmolH_2/g de DQO em cultivo misto e condição termofílica (55°C), verificando que houve um decréscimo na produção de hidrogênio de 2,31 para 0,44 mmolH_2/g de DQO com o aumento da concentração de vinhaça de cana-de-açúcar (2 a 12 g DQO L^{-1}). Ainda, Buitrón & Carvajal (2010), com o emprego de vinhaça de tequila, relataram uma velocidade de produção de hidrogênio de 50,5 mL/L/h quando o reator foi alimentado com uma DQO de 3 g/L, em cultivo contendo culturas mistas. E Albanez et al. (2016) utilizando uma mistura de vinhaça e melaço (67-33%) obtiveram 6,2 mmol H_2 /g de DQO com consórcio microbiano proveniente efluente de criação de aves.

4. RESULTADOS E DISCUSSÃO

A figura 4 apresenta o fluxograma experimental da realização deste trabalho: origem do inóculo de lodo granular; métodos de pré-tratamento térmico do inóculo, concentrações de vinhaça utilizada, ensaios de produção de hidrogênio (consórcios microbianos e microrganismos isolados), avaliação da produção de ácidos graxos voláteis, consumo de carboidratos totais e redução de DQO, análises microbiológicas, isolamento, microscopia e análises moleculares (PCR-DGGE; quantificação da Fe-hidrogenase; sequenciamento de alto desempenho 16S e 18S rRNA).

Os resultados serão apresentados na forma de 2 capítulos. O primeiro artigo intitulado "Effect of the inoculum heat treatment in the production of hydrogen and volatile fatty acids by dark fermentation of sugarcane vinasse" teve como objetivo avaliar a influência de três métodos de pré-tratamento térmico do inóculo, o efeito do pH inicial e da concentração de vinhaça em ensaios em batelada na geração de hidrogênio e ácidos graxos voláteis.

O segundo artigo intitulado "Variation of the prokaryotic and eukaryotic communities after different methods of thermal pretreatment of the inoculum in the hydrogen production process" teve o objetivo de comparar a diversidade dos consórcios microbianos após três métodos de pré-tratamento térmico, com diferentes valores iniciais de pH (5, 6 e 7) na produção de hidrogênio e ácidos graxos voláteis utilizando a vinhaça de cana-de-açúcar como substrato.

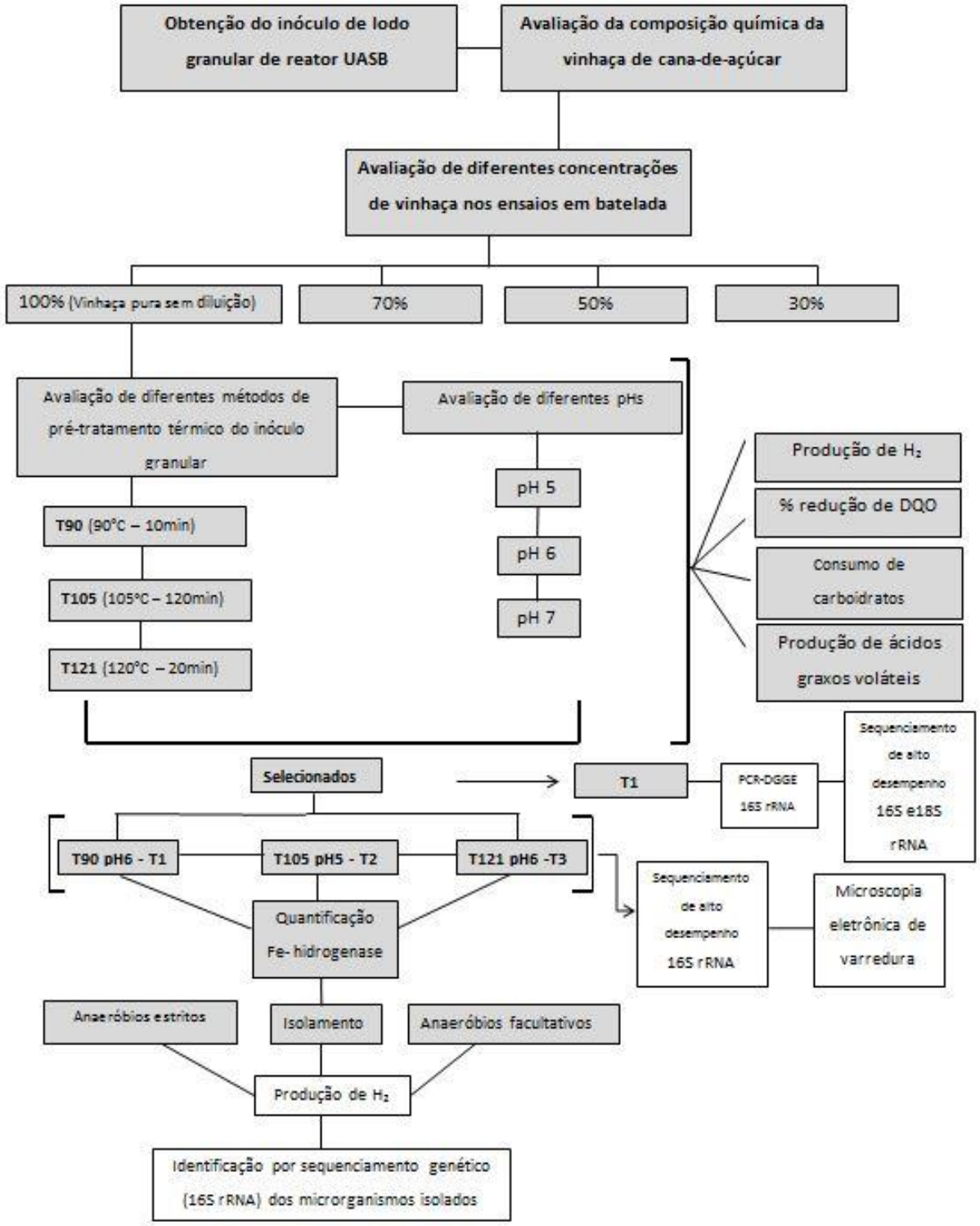

Figura 4: Fluxograma experimental na realização dos ensaios em batelada de produção de hidrogênio e ácidos graxos voláteis utilizando vimhaça como substrato.

4.1 Capítulo 1

Effect of different heat treatments of inoculum on the production of hydrogen and volatile fatty acids by dark fermentation of sugarcane vinasse

Abstract

Vinasse the main residue of bioethanol production, can be used as a substrate in biological process to obtain value-added products. However, it is necessary to use a microbial consortium with high capacity to produce hydrogen (H2) and volatile fatty acids (VFA). There is no consensus on the best inoculum pretreatment to eliminate hydrogen-consuming bacteria. Thus, the present study evaluated the influence of three methods of heat pretreatment of the inoculum: T1=90 °C/10 min; T2=105 °C/120 min; T3=121 °C/20 min for the production of H2 and VFA using vinasse as substrate. The effect of the concentration of vinasse and the initial pH (7, 6 and 5) were also evaluated. The best hydrogen production (821.34 mL) and yield (4.75 mmol H2.g-1COD) was obtained using undiluted vinasse at pH 6 and T1 pretreatment. The highest number of copies of the Fe-hydrogenase genes confirms the higher H2 production. The presence of Clostridium and facultative anaerobic microorganisms Bacillus and Enterobacter in the microbial consortia were confirmed by isolation and PCR-DGGE. The highest production of VFA was obtained at pH7 and T3 pretreatment. This study showed that dark fermentation could be driven by the inoculum pretreatment and pH selecting different process either for the production of H2 or VFA.

Keywords: hydrogen production; pretreatment of the inoculum, vinasse; VFA production; microbial community; Fe-hydrogenase qPCR.

1. Introduction

The production of ethanol from sugarcane is an efficient alternative to replace fossil fuels in tropical countries. Brazil is the world's largest producer of ethanol from sugarcane, reaching a record production of 33.1 billion liters of ethanol in 2018/2019 [1]. However, for each liter of ethanol, 12-15 liters of vinasse are generated [2]. Vinasse is a dark brown liquid with acid pH (3.0-5.0), has high organic matter (22 to 45 $gCOD.L^{-1}$) and a polluting power of 100 times more than domestic sewage [3]. Currently, vinasse is mainly used as a fertilizer in cane cultivation itself [4]. However, long-term use may

cause desertification and salinization of soil and decrease of agricultural productivity [2]. Therefore, the rational use of vinasse in anaerobic digestion for the production of hydrogen and volatile fatty acids can be a sustainable alternative to the use of this residue.

Hydrogen (H_2) is a clean and promising form of energy for the coming decades, as it produces only water vapor as the product of its combustion and does not result in the formation of CO, CO_2 and hydrocarbons that contribute to the greenhouse effect [5]. Recently, the production of volatile fatty acids by fermentation using agro-industrial waste has been gaining prominence due to its wide range of use in the pharmaceutical, food, chemical and valuable raw materials industries for products such as biogas [6,7].

Biological processes for the production of hydrogen and volatile fatty acids can be obtained by pure cultures or by microbial consortia. In large-scale applications, consortia are more advantageous because sterilization of the culture medium is not required and a wide variety of residues are used as the substrate [8]. Another critical aspect of the use of consortia is the use of natural sources of inocula, such as soil samples, anaerobic digestion sludge, sewage wastewater, and domestic landfill, among others [9].

In the environment, the microbial consortia are evolutionarily associated with the degradation and production of several compounds, where each microorganism has a role in this process, either in substrate hydrolysis, the degradation of organic compounds, in oxygen consumption, and participating in the phases of acetogenesis, acidogenesis, hydrogen production and methanogenesis. Several microorganisms are present in consortiums and are capable of producing hydrogen, with the predominance of strict anaerobic bacteria belonging to the genus *Clostridium*, such as *C. butyricum*, *C. pasteurianum*, *C. paraputrificum* and *C. bifermentants* [10], other facultative anaerobic microorganisms belonging to the Enterobacteriaceae family, such as *Enterobacter cloacae* [11] and *Klebsiella pneumonae* [12], as well as species of the genus *Bacillus* [13,14].

The H_2 produced by these microbial consortia can be used as a fuel, but when retained in the reactor it acts as an electron donor to produce both acetic acid (homoacetogenesis) and methane (hydrogenotrophic methanogenesis) [15] damaging the recovery of the gas. One of the ways to avoid this effect is the application of inoculum pretreatment methods, such as heat treatment (>90°C) to suppress the activity of hydrogen-consuming bacteria (methanogenic archaea), selecting endospore *Clostridium*, *Bacillus* and *Thermoanaerobacterium* [16].

The objective of this work was to evaluate three methods of heat pretreatment of the inoculum to produce hydrogen and volatile fatty acids at different initial pH values, using vinasse as a substrate. The microbial composition of the biomass after each pretreatment was also evaluated.

2. Material and methods

2.1. Substrate

Sugarcane vinasse from the ethanol industry Guarani - Andrade Industrial Unity (Pitangueiras, SP, Brazil) was used and kept in a freezer (-20°C). Before use, the vinasse was centrifuged at 2,000 g for 20 min and the supernatant was used in the experiments. For the tests, the vinasse was diluted in distilled water at concentrations of 30%, 50%, 70% and 100% undiluted pure vinasse. Vinasse has not been enriched with *another type of nutrient or carbon source*.

2.2. Inoculum pretreatments

The microbial consortia employed was a granular sludge from the methanogenic anaerobic reactor (UASB) treating the effluent the vegetable oil industry (Esteio/RS, Brazil). Three different heat treatments were tested: 90°C for 10 min (water bath) (T1) [17]; 105°C for 2 h (T2) [18]; and 121°C for 20 min (autoclave) (T3) [19].

2.3. Batch fermentation assays for H_2 and volatile fatty acids production

Fermentation experiments were performed to test the production of H_2 and VFA in different conditions. Two sets of experiments were performed; a first set was performed to determine the effect of the concentration of vinasse. For this experiment, the inoculum used was thermally pre-treated at 90°C for 10 min (water bath) (T1) and the initial pH was set at 6. The second sets of experiments were performed to test the impact of the inoculum pretreatments and the effect of the initial pH (5, 6 and 7). The initial pH was adjusted using 1M HCl or 1M NaOH solution (APHA, 2005). For these assays, the vinasse with no dilution (100%) was used. All the tests were carried out in batch laboratory scale in glass vials of 600 mL containing 300 mL of headspace and 300 mL of vinasse culture medium in the different concentrations, with the addition of approximately 3 $g.L^{-1}$ of total solids (TS) of the inoculum after the different heat treatments. The flasks were shaken (Ethik Technology) at 140 rpm, 37°C, and in the absence of light during the experimental period. To guarantee the anaerobiosis, N_2 gas (99.993%) (Air products) was injected in the liquid medium and the headspace for 10 min. All assays were performed in triplicate. During the incubation, the H2 production was determined in the headspace, and liquid samples

were taken to determine the concentration of VFA, chemical oxygen demand (COD) and sugars. pH final was determined using a pHmeter (Denver Instrument). Samples from the biomass (5 mL) were taken at the end of the experiments and from the inoculum without treatment and stored at -20 ^{o}C for DNA extraction.

2.4. Chemical analysis

The concentration of the H_2 in the biogas was measured by gas chromatography in gas samples taken from the headspace of the batch tests. A chromatographic column CarboxenTM 1006 PLOT Capillary Column (30 m × 0.53 mm) in a Dani Master – Automatic Sample AS chromatographer equipped with thermic conductivity detector (TCD) was used. Ultra-pure nitrogen gas was used as the carrier gas at 6 mL min^{-1} flow. The injector temperature was 100 ºC, the detector temperature was 230 ºC and the column temperature was 40 ºC. For the preparation of the calibration curve, volumes of 10, 25, 50, 100, 150, 200 and 250 μL of pure hydrogen gas were injected.

The concentration of VFA (acetic, propionic, butyric, isobutyric, valeric and isovaleric acids) and ethanol were determined in liquid samples using a gas chromatograph (GC/MS, Shimadzu - QP2010 Ultra) equipped with a DN - FFAP column (30 m x 0.32 mm x 0.25 μm) with Flame Ionization Detector (FID), with Helium as the carrier gas, as well as synthetic air and nitrogen as auxiliary gases. The column temperature was 100 °C for 5 min, increasing by 7 °C min^{-1} to 200 °C. The injector and detector temperatures were 200 °C and 250 °C, respectively. The samples were previously centrifuged (Vision – CE) at 10 min for 10,000 rpm and filtered in a membrane of 0.22 μ.

The analysis of the chemical composition of the vinasse, the pH measurements and the COD analyses and total solids were performed according to Standard Methods [20]. The concentration of total carbohydrates was determined as described by Dubois et al. [21].

2.5. Experimental data fitting

The modified Gompertz equation was used to estimate the kinetic parameters for hydrogen production. The H_2 volume obtained during the tests versus time was treated with the program Statistica 7 and modeled as Eq. (1), to obtain the kinetic parameters Rm, Hmax and λ.

Equation 1: $$P = H_{max} \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\}$$

Where, P = cumulative volume of H_2 in the tests, Hmax = maximum volume of H_2 (mL), Rm = maximum rate of H2 production (mL / h), λ = phase lag (h), and t = tests (h).

The volume of hydrogen was converted to mmol by applying the ideal gas equation (PV = nRT), where P is the atmospheric pressure in Caxias do Sul (0.918 atm), V is the volume of H_2 (liters), *n* is the number of mols of H_2, and R is the universal ideal gas constant (0.082 atm L. $^oK^{-1}.mol^{-1}$), and T is the temperature in the tests (^oK).

The VFA yield was calculated using COD influent (measured at the beginning of the experiment), according to the equations: VFA yield: mg acid / gCOD.

2.6 Statistic tests

The data generated by the hydrogen and VFA production tests were treated by the software GraphPad Prism 5.0 and submitted to analysis of variance and Tukey test ($p \leq 0.05$).

2.7. Microbiological analysis

2.7.1. Denaturing gradient gel electrophoresis analysis (DGGE)

Samples from the biomass were de-frozen and the DNA was extracted with the commercial Soil DNA Isolation Kit (Mo Bio Laboratories), following the manufacturer's protocol. Amplification of partial 16S rRNA genes for DGGE analysis was performed with the primers 968F (with GC clamp) and 1392R, using the protocol and reaction conditions described by Nielsen et al. [22].

PCR products were separated on a polyacrylamide gel with a denaturing linear gradient ranging from 15% to 55%. The gel was stained with SYBR Safe DNA gel stain (Invitrogen). The images of the bands patterns were captured with a transilluminator (Vilber Loumart). Selected bands were excised, eluted in 50 μL of ultrapure water, and incubated at 4 °C overnight. The DNA was then re-amplified with the same primer pair, excluding the GC-clamp, as described. The sequence of the DNA retrieved from the bands were obtained as will be described.

2.7.2. Isolation of microorganisms

The microorganisms were isolated from biomass samples taken at the end of the batch experiments showing the better performance with the different heat treatments (T1, T2 and T3). Isolation was performed by streaking the samples with a loop in microbiological dishes containing PYG culture medium $g.L^{-1}$ (glucose 10, meat extract 5, yeast extract 5, peptone 5) supplemented with 13% agar (HiMedia) at pH 7, thioglycolate (HiMedia) and MacConkey selective medium (Himedia). The plates were incubated in anaerobic jars at 37 °C. The isolated microorganisms were purified and stored in glycerol 30% in ultra-freezer (-80 °C).

2.7.3. Production of H_2 of the isolates

The capacity to produce H_2 was tested for the isolated microorganisms in batch assays performed as explained before but using 60 mL glass vials containing 30 mL of headspace and 30 mL of a liquid medium composed by vinasse in a dilution of 50%. The media were inoculated with one volume of culture, corresponding to the reading of 1 O.D. (optical density) in a spectrophotometer (Molecular Devices) at 600 nm.

2.7.4. Identification of bacterial isolates by 16S rRNA gene sequence

DNA extraction from the isolated microorganisms was performed with the HiMedia HIPura™ Bacterial genomic DNA Purification Kit (HiMedia Laboratories) according to the specification of the suppliers. 16S rRNA genes amplification was performed by Polymerase Chain Reaction (PCR) with Phusion™ High-Fidelity PCR kit (Finnzymes) according to the manufacturer's protocol using the DNA extracted from the isolates. The primers and conditions used as described by Poleto et al. [23].

2.7.5. 16S rRNA gene sequencing from isolates and DGGE bands

The 16S rRNA gene PCR products obtained from the isolated microorganisms and the DGGE bands were purified using Exonuclease I (Amersham Biosciences, 2500 U) and Shrimp Alkaline Phosphatase (Amersham Biosciences, 5000 U) enzymes according to the manufacturer's protocol. Capillary sequencing was performed using ABIPRISM 3130 xl automatic sequencer (Applied Biosystems, USA). The sequences were compared to other sequences deposited in the National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov) database using a nucleotide BLAST tool (BLASTn) (Edgar 2010). Sequences from this study were deposited in NCBI under the following accession numbers: MH460645, MH464164, MH469832, MH469851, MH477635, MH477725, MH481532, MH504164, MH504165, MH509479, MH509759, and MH511147.

2.7.6. Quantification of Fe-hydrogenase genes by quantitative PCR

Quantitative PCR (q-PCR) was used to quantify the proportion of hydrogen-producing bacteria that contained the enzyme Fe-hydrogenase gene in DNA samples from the biomass of the batch assays experiments. The number of copies was determined using the method described by [24]. The PCR reaction mixture consisted of 10 µL of SYBR Green mix (Rotor Gene SYBR Green RT-PCR kit, Qiagen Inc., Valencia, CA, USA), 1 µL of each primer and 8 µL of the DNA template. A calibration curve was constructed using 10-fold dilutions of a Fe-hydrogenase gene PCR product from a Fe-hydrogenase clone as standard as was explained by Perna et al. [25]. The amplification reactions were performed in a Rotor Gene 6000 (Corbett Research, Sidney, Australia). The reaction was performed in duplicate, and the

average and standard deviation were determined. DNA was quantified using a fluorometer (Qubit 2.0, Invitrogen, Carlsbad, CA, USA).

3. Results and discussion

3.1. Effect of the vinasse concentration in the production of H_2 and VFA

The concentration of the vinasse positively affected the production of hydrogen, the highest cumulative production (821.34 mL) in the experiments performed with the vinasse at 100% with no dilution (Figure 1). The other concentrations tested (30%, 50% and 70%) presented lower hydrogen production, of 128.76, 444.26, 511.04 mL, respectively. Similar results were found in the work performed by Lazaro et al. [3], the authors also verified an increase in the hydrogen production with the increase of the vinasse concentration in mesophilic tests.

3.2. Vinasse characteristics

The chemical composition of the medium used in the experiments presented in Figure 1 was evaluated to determine the nutrients available in the vinasse. The analysis showed the presence of nutrients required to microbial growth, such as nitrogen, calcium, iron, magnesium, higher concentration of potassium (>2,500 $mg.L^{-1}$), manganese and phosphorus (Table 1). According to bibliography, the amount of iron detected in the vinasse prior to fermentation is sufficient for the metabolism of microorganisms such as *Clostridium pasteurianum* and 10 $mg.L^{-1}$ is the optimum concentration for the production of hydrogen for this bacterium [26].

The vinasse, as well as containing nutrients, has a residual content of sucrose, trehalose [27]. Analyzes performed carried out by our research group detected the following sugars present in vinasse: cellobiose (0.052 $mg.L^{-1}$), xylose (0.258 $mg.L^{-1}$), glucose (0.354 $mg.L^{-1}$) and arabinose (0.030 $mg.L^{-1}$). Therefore, the vinasse was used in experiments without the addition of other minerals or carbon sources, which implies a reduction of industrial scale fermentation costs.

However, the chemical composition of vinasse depends on the characteristics of the soil, the sugarcane variety, the season of the year in which the crop is harvested, and the industrial process used to produce ethanol [3,4].

3.3 Production of H_2 using different inoculum pretreatments and different initial pH

Batch fermentation experiments were carried out with vinasse at a concentration of 100%, at different pH values and comparing three methods of heat pretreatment of the inoculum (Table 2). The results showed that both pH and pretreatment of the inoculum affect the production of H_2. The pretreatment T1 presented the best results compared with the other pretreatments with values of Hmax=821.34 mL at initial pH 6. The pretreatment T3 presented intermediate values of 697 and 687 mL at an initial pH of 6 and 5 respectively. The pretreatment T2 presented the intermediate values of 687.58 mL when the experiment was performed at pH 5, but lower values when the experiments were done at higher pH values. The highest values (2.73 $LH_2.L^{-1}$, 821.34 mL), were similar to the values observed by Sydney et al. [2], using vinasse supplemented with cane juice (2.25 $LH_2.L^{-1}$ of culture medium). It has to be considered that in our work the vinasse was used with no dilution and without the supplementation of culture medium. However, Ferraz Júnior et al. [28], evaluating different types of materials as support in the APBR (acidogenic packed-bed reactor), obtained a maximum of 28.1 mL $H_2.L^{-1}$ using low-density polyethylene as a support in the reactor and pure vinasse as substrate. Moreover, the maximum hydrogen production found in our work P = 29 mmol (821.34 mL) is similar to that found by [3] (P = 28.4 mmol) using 12 g $COD.L^{-1}$ of vinasse supplemented with nutrients and vitamins.

With respect of the effect of the initial pH in the production of H_2, it is clear that depending on the inoculum pretreatment, the H_2 production is more or less affected by initial pH (Table 2). In the experiments performed with the inoculum with pretreatment T3 similar values of the potential of H2 production (662 and 697 mL) at pH 5 and 6 were obtained, respectively, and lower value at pH 7 (477 mL). While the experiments performed using the inoculum with T2 pretreatment showed an important negative effect of the initial pH used in the experiments, with values of 687 mL at pH 5, 376 mL at pH 6 and 57 mL at pH7. This result suggests that with this pretreatment non hydrogen producing fermentative microorganisms were selected and these microorganisms were highly affected by pH. On the other hand, the assays performed using the inoculum pretreatment T1 presented the higher production at pH 6 and lower values at pH 5 and 7.

The highest consumption of the carbohydrates (57%) was observed in the assays performed at initial pH 6 and pretreatment of inoculum T2 followed by pretreatments T1 and T3, both assays presented a 47% of substrate removal (Table 2).

In all experiments, an extensive lag phase (λ) was observed (Table 2), where hydrogen production occurred after 13h of fermentation. The lag phase was directly affected by the initial pH value. When initial pH was set to 5, the largest lag phase occurred using the inoculum with pretreatment T2 (57 h), followed by T3 at initial pH 5 and pH6 (31 and 34 h). Zhang et al. [29] also verified a long lag phase of 72 h at pH 5 in a thermophilic batch reactor containing starch as substrate. Also, the rate of hydrogen production (R_m) was between 8 and 98 $mL.h^{-1}$ (Table 2), which was higher than those obtained by [30] for vinasse (23.16 $mL.h^{-1}$) and sewage (8 $mL.h^{-1}$).

The experiments performed at pH 7, presented the lowest lag phase (13-23 h) and also the lower hydrogen production for the three pretreatments evaluated (Table 2), favoring the production of volatile fatty acids. The initial pH is one of the main parameters that influence the production of hydrogen since it directly affects the microbial metabolic pathway and the production of hydrogen [31]. Low initial pH (<5.0) or high pH (>6.5) may result in the inhibition of hydrogenase activity and a consequent decrease in hydrogen production [32]. In the literature, there is no consensus regarding the optimal initial pH value for hydrogen production, since it depends on other factors such as type of inoculum, substrate, and temperature, among others [33].

The rate of removal of COD in the production of hydrogen (Table 2) was between 10 and 25%, which was also found by Albanez et al. [34] using vinasse as a substrate. This was expected, considering that the carbonaceous matter is oxidized to organic acids and not completely mineralized to CH_4 and CO_2, as occurs when the anaerobic digestion is complete. To increase this efficiency, the process must be carried out in two stages: the first with hydrogen production followed by methanogenic production from the same substrate, where up to 80% of COD removal can be achieved [35]. Low organic matter removal efficiencies do not represent an environmental problem, since the acidogenic reactor for hydrogen production would be the first unit in a wastewater treatment plant and the effluent from this food unit would be a second stage, which could be as a methanogenic anaerobic reactor with the effective reduction of COD and methane production [30]. On the other hand, the VFA produced could be retrieved for the further valorization of the vinasse.

The highest yield found in this work was 4.75 $mmolH_{2.}g^{-1}COD_{influent}$ (Table 2 and 3), which was higher than the higher value found by Lazaro et al. [3] (2.23 $mmolH_2g^{-1}COD_{influent}$), and lower than the yields seen by [34] 6.2 $mmolH_2g^{-1}COD_{influent}$ with vinasse/molasses (67-33%) without substrate supplementation. However, the maximum yield of hydrogen (24.94 $mmolH_{2.}g^{-1}COD_{influent}$) was obtained

by the work of Fernandes et al. [30] in a batch reactor using anaerobic methanogenic sludge (Table 3). It is worth mentioning that hydrogen yields differ significantly from one study to another, as this also depends on the reactor volume used, the reactor design, the inoculum and also the characteristics of the vinasse used, regardless of whether or not it is enriched with other nutrients.

The methods of heat pretreatment have been widely described in the literature [18] for the elimination of non-spore forming methanogenic bacteria, since hydrogen-producing bacteria, such as most *Clostridium*, can form protective spores and survive extreme conditions. The heat pretreatment conditions reported in the literature range from 80 to 121°C and exposure time from 15 to 120 min [19, 36]. During the experiments of this work, only H_2 and CO_2 were produced, the production of CH_4 was not observed, demonstrating that the three methods of heat pretreatments used were useful in the elimination of methanogenic archaea. However, the methods were not effective in the control of other hydrogen consuming microorganisms, such as homoacetogens (consume hydrogen to produce acetic acid) and propionic producing bacteria. Guo et al. [37] evaluated three methods of pretreatment of the inoculum and verified that the sterilization treatment (121°C – 20 min) produced the highest hydrogen yield (15.02 $mLH_2.gCOD^{-1}$) in comparison to microwave and ultrasound pretreatments. Mu et al. [38] compared three pretreatment methods and indicated that heat pretreatment (102°C for 90 min) produced the highest amount of hydrogen (1,504 mL) when compared to two other pretreatment methods (acid and alkaline) of the inoculum.

In this study, heat treatment T1 (90 ° for 10 min) had the best performance in hydrogen production (Table 2). These results are very important as is the treatment with lower temperature and time, which results in lower costs of the application. Some studies have reported a substantial increase in H_2 yield after heat treatment at low temperatures [39]. Low temperatures or brief pretreatment time may not be adequate to inhibit many H_2 consumers, while very high temperatures and long treatments may lead to loss of H_2 producer's activities [39]. For example, Baghchehsaraee et al. [40] obtained an H_2 yield of 1.6 mol/mol glucose for thermally treated activated sludge (65 °C for 30 min) being 530% larger than the control. These authors observed that increasing the temperature (80 °C to 95 °C) led to decreased yield. In contrast, Alibardi et al. [41] evaluated different pretreatment times (0.5 to 4 h) of granular sludge heat treatment at 100 °C and obtained the maximum H_2 yield of 2.14 mol / mol glucose for the sludge pretreated for 4 h. Wang and Wan [42] obtained the maximal hydrogen yield of 221.5 $mL.g^{-1}$ glucose using digested sludge pretreated by heat-shock (100 °C for 15 min) in comparison with others

pretreatment methods (acid, base, aeration and chloroform). However, most of the work was performed using glucose and few studies have been conducted using complex substrates such as vinasse.

3.4. Volatile fatty acids production

The initial pH in the fermentation positive affect the production of VFA with higher production in the assays performed at pH 7, but this effect also depends on the pretreatment used for the inoculum (Figure 2). For the experiments conducted at pH 6 and with the inoculum with pretreatment T1, in which the highest hydrogen production was observed, the production of butyrate was favoured (380 $mg.L^{-1}$). This indicates that the production of H_2 was linked to the butyrate fermentation. For the same pretreatment (T1) but at pH 5, a predominance of propionic acid (213 $mg.L^{-1}$) was observed, while at pH 7 there was a predominance of the acetic acid (490 $mg.L^{-1}$) (Figure 2a), evidencing that pH is the key to determine the metabolic pathway that will be favoured during the process. Khan et al. [43] also found acetic acid predominance at pH 7, while the maximum concentration of propionic acid was obtained at pH 6 in an anaerobic membrane bioreactor treating synthetic wastewater. In the experiments performed with pretreatments T1 and T3 at pH 7, higher production of all evaluated acids (mainly acetic, butyric and propionic acid) was observed (Figure 2a and c). Similar to that found by Chen et al. [44], they obtained an increase in the concentration of VFAs in alkaline conditions from waste activated sludge. Possibly, at pH 7, the homoacetogenic microorganisms and propionic acid-producing microorganisms that consume hydrogen were favoured [45]. At pH 6, the butyric route was favoured in the three pretreatments. The highest concentration of the acids was obtained in the assays performed with the inoculum with T3 pretreatment while the lower values were obtained using the T2 pretreatment. Higher production of valeric acid (95 $mg.L^{-1}$) was observed in the experiments performed using the T2 pretreatment, and the total acid concentrations were similar in all the experiments performed at different pH values (Figure 2b). These results indicate that both pH and inoculum pretreatment were important factors that affected the performance of the fermentation process for hydrogen production and yield of individual volatile fatty acid, selected distinct metabolic routes (Table S1, Supplementary material).

The preferred metabolic pathways for hydrogen production are acetic and butyric [46]. The butyric metabolic pathway was found in 50% of all *Clostridium* isolates [18]. The acidogenic saccharolytic microorganisms ferment carbohydrates and produce high amounts of hydrogen, such as

Clostridium butyricum, which produces 2 mol of butyric acid as the main fermentation product, along with 4 moles of H_2 and CO_2 and according to Equation 2 from sucrose [18, 46].

Another metabolic pathway observed in the experiments was the propionic acid fermentation. According to equation 3 [47], the production of propionic acid from glucose is unfavorable for the production of hydrogen, since 2 moles of hydrogen are consumed for each mole of propionic acid produced, leading to low hydrogen production.

Equation 2: $C_{12}H_{22}O_{11} + H_2O \rightarrow 2CH_3CH_2CH_2COOH + 4CO_2 + 4H_2$

Equation 3: $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2CH_2COOH + 2H_2O$

On the other hand, in the experiments with low hydrogen production (T3 and T1 both at pH 7), there was a high VFAs yield, being 79.76 mgVFA.g^{-1}COD and 44.01 mgVFA.g^{-1}COD, respectively (Table 2) indicating that the organic matter was potentially convertible into VFAs, revealing the potential of sugarcane vinasse in the production of these acids. Morgan-Sagastume et al. [48] also obtain high VFAs yield of 15–20 $gVFA_{COD}L^{-1}$ from sludge fermentation with the pretreatment of high-pressure thermal hydrolysis. Volatile fatty acids can become valuable substrates for more refined final products [49]. They are used for various purposes in the chemical, food and pharmaceutical industries [50]. Ethanol production was not detected in these experiments, possibly because a deficient production was not detected by the method used to determine the VFA.

3.5. Microbial community analysis

Samples from the assays with the higher production of H_2 using the three different inoculum pretreatments were selected (T1 pH6; T2 pH 5 and T3 pH6) and the microbial community was analyzed by 16S rRNA gene DGGE. The DGGE profiles indicated that the different pretreatment selects different microbial communities. The microbial community from the assay performed with the pretreatment T1, which presented the higher production of H_2, were composed mainly by *Clostridium*. While in the samples from the experiments performed with the other pretreatments (T2 and T3) the bands presenting sequences related to *Clostridium* sequences were not so intense and other bands with sequences related to sequences from other microorganisms with lower H_2 production capabilities were observed (Figure 3).

A high proportion of *Clostridium* was observed in the samples from the assays with higher H2 production. This genus was described in the literature as a hydrogen producer and is probably the main one involved in this production [51,52]. In the samples taken from the experiments performed with T2 and T3 pretreatments, band 2 presented high intensity. The sequences retrieved from this band presented 99% similarity with the 16S rRNA gene sequence from *Bacteroides xylanolyticus*, which was also related to hydrolysis, acidogenesis [53] and hydrogen production [54].

According to Li and Yu [55], the productivity of H_2 and the structure of the inoculum granule are highly-dependent on the microbial species that compose it. The genus *Clostridium* contains strict anaerobes, which are known to be excellent H_2 producers [56], but are extremely vulnerable to oxygen. However, other genera such as *Enterobacter* and *Klebsiella*, have a lower yield in hydrogen production, but can consume oxygen and help to maintain an anaerobic environment within granules [57].

3.6. Fe-Hydrogenase quantification

To know if the different inoculum pretreatment selects microorganisms with high capacity to produce hydrogen, the Fe-hydrogenase genes were quantified by q-PCR in the DNA from samples taken from the biomass at the end of the fermentation assays. The samples with higher production of hydrogen and the initial inoculum without any pretreatment were included in the analysis. The results of PCR quantification showed that samples obtained in the assay with vinasse 100% and the inoculum with T1 pretreatment at initial pH 6 presented the highest number of copies per mL of the gene Fe-hydrogenase (Fig. 4). This is correlated with the sample of the highest hydrogen production (Hmax= 821.34 mL), followed by the sample of the assay performed with the pretreatment T3 pH6 (Hmax = 697.68 mL). The initial inoculum sample, before fermentation, and the samples from the other assays (pretreatment T2 at pH6 and pH7), presented lower number of copies of Fe-hydrogenase, which was expected since the hydrogen production was low in these assays.

The quantification of Fe-hydrogenase is an important parameter in the monitoring of the fermentation process since the hydrogenase enzymes are directly involved in H_2 metabolism and are found in anaerobic bacteria such as *Clostridia*, *Thermotoga* and *Desulfovibrio* [58]. The higher values obtained in this work were similar to the higher values obtained in the work of Castelló et al. [59] using the same method. These authors determine the Fe-hydrogenase copy number in samples taken from a CSTR bioreactor fed with cheese whey, the highest values observed by the authors were 10^8 Fe-

hydrogenase copy numbers/L suggesting that this value is representative of a suitable d hydrogen producing microbial community.

3.7. Isolation and identification of the microorganisms from the fermentation assays

Samples were taken for the isolation at the end of the batch assays performed with the different inoculum pretreatments. According to 16S rRNA gene sequence analysis, all the isolates belong to species from facultative anaerobic microorganisms: *Bacillus cereus*, *B. amyloliquefaciens*, *B. toyonensis*, *B. subtilis*, *B. nakamurai*, *Lysinibacillus boronitolerans*, *Enterococcus faecalis*, *E.asburiae*, *E. tabaci* and *E. muelleri* (Table 5). These microorganisms are present in the microbial consortia and may contribute in some way to the production of hydrogen (Table 4), but few studies in the literature described these microorganisms as direct producers of this gas.

Among the number of microorganisms isolated, 68% belong to the Bacillaceae family (Table 4), because they are spore-forming species, survive extreme conditions and compete with other microorganisms, whilst having versatile enzymatic activities, such as amylolytic activity, which can be very useful for the conversion of food waste and other various bio-wastes to hydrogen.

Species of the genus *Bacillus* and *Lysinibacillus* have been reported to have hydrolytic activities, multiply rapidly and secrete large amounts of proteins. Therefore, they are attractive species for the industry. These properties are beneficial for industrial applications bacilli, which contribute to 50% of the enzyme market [60].

Interestingly, non-sporulating microorganisms of the genus *Enterobacter* were isolated in the pretreatments at 105 °C and 120 °C (Table 4). The same was also found by Iyer et al. [61], who identified Enterobacteria in the inoculum after heat treatment of 105 °C for 2 h, indicating that the thermal treatment does not destroy all vegetative cells before inoculation in the bioreactor. In our work, these microorganisms are grouped and protected within the granular inoculum, allowing that tolerate heat stress [41].

The microorganisms observed by isolation were not detected by the DGGE analysis (Fig. 3) this could be due to the limitations of DGGE methods in which only the most abundant microorganisms were detected or by the limitations of the isolation techniques. Contrarily, high coverage of the microbial communities was achieved in work performed by Fuentes at al. [62]. In this work the authors used different strategies to isolate the fermentative microorganisms present in two samples from a hydrogen

producing reactor, they also study the microbial composition by 16S rRNA gene pyrosequencing analysis. The higher coverage found in the work of [62] could be due to the higher coverage of the pyrosequencing method compared with the DGGE method and also for the different techniques applied for the isolation. In particular, for the isolation of *Clostridium* the authors propose the heat treatment of the sample before cultivation, this could be an essential step in the procedure to isolate *Clostridium*.

3.8. Production of hydrogen by the isolates

The capacity to produce hydrogen by the isolates were tested in a medium containing vinasse diluted at 50%. From 22 strains tested 10 presented production of hydrogen in this medium (Table 4 and Figure S1, Supplementary material). The higher production corresponds to the strains classified within the family Enterobacteriaeace, with the highest values for *E. muelleri* (1,494 $mLH_2.L^{-1}$) and *E. tabaci* (1,119 $mLH_2.L^{-1}$); the exception was *E. faecalis* (200 $mLH_2.L^{-1}$) (Figure S1, Supplementary material). For the strains classified within the *Bacillus* genus, the hydrogen production of *B. subtilis* (1,200 $mLH_2.L^{-1}$) was similar to the production of the strains classified as Enterobacteriaceae.

For the other strains classified within the *Bacillus* genus, the average production of hydrogen was around 400 $mLH_2.L^{-1}$ (Figure S1). The hydrogen production obtained by the isolates (1.49 $LH_2.L^{-1}$) was lower than that obtained by the microbial consortia (2.73 $LH_2.L^{-1}$). This difference indicates that in the consortium probably other microorganisms not isolated with the methods applied to play a vital role in the hydrogen production.

Several studies have described the potential of *Enterobacter* species in the production of hydrogen from different substrates [63, 64]. These microorganisms can be associated in co-cultures or even added to the bioaugmentation of microbial consortia to increase the fermentative production of hydrogen, favoring the consumption of oxygen and making the environment favorable for strict anaerobes.

Also, the isolated microorganisms can be used to study the causes of the instability of hydrogen production. For example, these strains can be used to study the toxicity of potassium, sulfate, phenolic compounds and melanoidins present in vinasse [34, 65].

The use of vinasse for the production of hydrogen and VFA has several advantages since it is a residue generated in high proportion from the production of bioethanol and it is still possible to obtain

additional energy from this process. This hydrogen produced may have several applications, including a power source for fuel cells of electric cars [30].

In this work, three different heat treatment of the inoculum were tested, according to the results obtained the better treatment for the production of hydrogen was the T1 (90 °C for 10 min). Using the inoculum with this pretreatment and a pH 6 a high production of H_2 was obtained from vinasse with no dilution and with any other extra supplement. These results are very important for the valorization of the vinasse. The liquid obtained after H_2 production can be further used in a second process in a methanogenic reactor, generating more energy and reducing the environmental impact of the disposal of this effluent. After anaerobic digestion, the effluent can still be used as a biofertilizer in several agricultural processes. On the other hand, the production of H_2 could be also complemented with the recovery of VFA.

But, it has to be taken into account that, the initial pH of the fermentation plays a key role in the process and, the optimum pH for H_2 is different than the optimum pH for VFA production.

4. Conclusions

The increase in vinasse concentration positively affected the hydrogen production and high production of hydrogen can be achieved with vinasse without dilution and with no other extra supplement. This result is very important for the valorization of the vinasse as the process will not require water and other extra supplies. It makes the process less expensive and environmentally friendly.

Both the pretreatment of the inoculum and the initial pH plays an important role in selecting different communities with different capabilities to produce H_2 and/or VFA. From the three pretreatments tested, the pretreatment T1 (90°C, 10 min) was the better to produce hydrogen at initial pH 6. While the pretreatment T3 was the most efficient to produce VFA at pH 7.

In the higher hydrogen production trials, the production of butyric acid was predominant. At pH 7 with the pretreatment T3, there was a reduction of hydrogen production, favoring the production of all volatile fatty acids, mainly acetic, butyric and propionic.

The highest number of copies of the Fe-Hydrogenase gene confirms the higher hydrogen production, indicating that this gene is an important marker to track the production of hydrogen.

In addition to the genus *Clostridium*, facultative anaerobic microorganisms such as *Bacillus* and *Enterobacter* have been shown to contribute to increasing the production of hydrogen.

These results show the influence of pretreatments of the inoculum on the conversion of the sugarcane vinasse in natura, producing hydrogen and fatty acids, and highlighting the environmental importance of providing energy from this residue of biofuel production generated daily in large quantities.

Acknowledgments

The authors thank Petrobras for their financial support and the University of Caxias do Sul and, Institute of Biological Research Clemente Estable, Uruguay.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. CONAB, National Supply Company (Companhia Nacional de Abastecimento). https://www.conab.gov.br. Accessed 25 August 2019.

2. Sydney EB, Larroche C, Novak AC, Nouaille R, Sarma SJ, Brar SK, Letti LAJ, Soccol VT, Soccol CR (2014) Economic process to produce biohydrogen and volatile fatty acids by a mixed culture using vinasse from sugarcane ethanol industry as nutrient source. Bioresour Technol 159:380-386. https://doi.org/10.1016/j.biortech.2014.02.042.

3. Lazaro CZ, Perna V, Etchebehere C, Varesche MBA (2014) Sugarcane vinasse as substrate for fermentative hydrogen production: The effects of temperature and substrate concentration. Int J Hydrogen Energy 39:6407-6418. https://doi.org/10.1016/j.ijhydene.2014.02.058.

4. Salomon KR, Lora EES (2009) Estimate of the electric energy generating potential for different sources of biogas in Brazil. Biomass Bioenergy 33:1101–1107. https://doi.org/10.1016/j.biombioe.2009.03.001.

5. Cheong DY, Hansen CL (2006) Bacterial stress enrichment enchances anaerobic hydrogen production in cattle manure sludge. Appl Microbiol Biotechnol 72:635-643. https://doi.org/10.1007/s00253-006-0313-x.

6. Atasoy M, Owusu-Agyeman I, Plaza E, Cetecioglu Z (2018) Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. Bioresour Technol 268:773–786. https://doi.org/10.1016/j.biortech.2018.07.042.

7. Strazzera G, Battista F, Garcia NH, Frison N, Bolzonella D (2018) Volatile fatty acids production from food wastes for biorefinery platforms: A review. J Environ Manage 226:278–288. https://doi.org/10.1016/j.jenvman.2018.08.039.

8. Lu Y, Slater FR, Mohd-Zaki Z, Pratt S, Batstone DJ (2011) Impact of operating history on mixed culture fermentation microbial ecology and product mixture. Water Sci. Technol 64:760–765. https://doi.org/10.2166/wst.2011.699.

9. Maintinguer SI, Sakamoto IK, Adorno MAT, Varesche MBA (2015) Bacterial diversity from environmental sample applied to bio-hydrogen production. Int J Hydrogen Energy 40:3180-3190. https://doi.org/10.1016/j.ijhydene.2014.12.118.

10. Sivagurunathan P, Sen B, Lin CY (2014) Batch fermentative hydrogen production by enriched mixed culture: Combination strategy and their microbial composition. J Biosci Bioeng 117:222-228. https://doi.org/10.1016/j.jbiosc.2013.07.015.

11. Maintinguer SI, Fernandes BS, Duart ICC, Saavedra NK, Adorno MAT, Varesche MBA (2008) Fermentative hydrogen production by microbial consortium. Int J Hydrogen Energy 33:4309-4317. https://doi.org/10.1016/j.ijhydene.2008.06.053.

12. Rossi DM, Costa JB, Souza EA, Peralba MCR, Samios D, Ayub MAZ (2011) Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. Int J Hydrogen Energy 36:4814-4819. https://doi.org/10.1016/j.ijhydene.2011.01.005.

13. Wang A, Gao L, Ren N, Xu J, Liu C, Lee DJ (2010) Enrichment strategy to select functional consortium from mixed cultures: Consortium from rumen liquor for simultaneous cellulose degradation and hydrogen production. Int J Hydrogen Energy 35:13413–13418. https://doi.org/10.1016/j.ijhydene.2009.11.117.

14. Motte JC, Trably E, Hamelin J, Escudié R, Bonnafous A, Steyer JP, Bernet N, Delgenès JP, Dumas C (2014) Total solid content drives hydrogen production through microbial selection during thermophilic fermentation. Bioresour Technol 166:610–615. https://doi.org/10.1016/j.biortech.2014.05.078.

15. Noori M, Saady C (2013) Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unresolved challenge. Int J Hydrogen Energy 38:13172–13191. https://doi.org/10.1016/j.ijhydene.2013.07.122.

16. Kraemer JT, Bagley DM (2007) Improving the yield from fermentative hydrogen production. Biotechnol Lett 29:685-695. https://doi.org/10.1007/s10529-006-9299-9.

17. Kim DH, Han SK, Kim SH, Shin HS (2006) Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrogen Energy 31:2158-2169. https://doi.org/10.1016/j.ijhydene.2006.02.012.

18. Khanal SK, Chen WH, Li L, Sung S (2004) Biological hydrogen production: efects of pH and intermediate products. Int J Hydrogen Energy 29:1123–1131. https://doi.org/10.1016/j.ijhydene.2003.11.002.

19. Ren NQ, Guo WQ, Wang XJ, Xiang WS, Liu BF, Wang XZ, Ding J, Chen ZB (2008) Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. Int J Hydrogen Energy 33:4318–4324. https://doi.org/10.1016/j.ijhydene.2008.06.003.

20. APHA (2005) Standart methods for the examination of water and wastewater. 21 st ed., Washington, DC.

21. Dubois MKA, Gilles JK, Hamilton PA, Rebers SF (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356.

22. Nielsen AT, Liu WT, Filipe C, Grady L, Molin S, Stahl DA (1999) Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. Appl Environ Microbiol 65:1251-1258.

23. Poleto L, Souza P, Magrini FE, Beal LL, Torres APR, Souza MP, Laurino JP, Paesi S (2016) Selection and identification of microorganisms present in the treatment of wastewater and activated sludge to produce biohydrogen from glycerol. Int J Hydrogen Energy 41:4374-4381. https://doi.org/10.1016/j.ijhydene.2015.06.051.

24. Fang H, Zhang T, Li C (2006) Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge. J Biotechnol 126:357-364. https://doi.org/10.1016/j.jbiotec.2006.04.023.

25. Perna V, Castelló E, Wenzel J, Zampol C, Fontes Lima DM, Borzacconi L, Varesche MBA, Zaiat M, Etchebehere C (2013) Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. Int J Hydrogen Energy 38:54-62. https://doi.org/10.1016/j.ijhydene.2012.10.022.

26. Liu G, Shen J (2004) Effects of culture and medium conditions on hydrogen production from starch using anaerobic bacteria. J Biosci Bioeng 98:251–256. https://doi.org/10.1016/S1389-1723(04)00277-4.

27. Fonseca BC, Guazzaroni ME, Reginatto V (2016) Fermentative production of H2 from different concentrations of galactose by the new isolate *Clostridium beijerinckii* Br21. Int J Hydrogen Energy 41:21109-21120. https://doi.org/10.1016/j.ijhydene.2016.09.110.

28. Ferraz Júnior ADN, Etchebehere C, Zaiat M (2015) Mesophilic hydrogen production in acidogenic packed-bed reactors (APBR) using raw sugarcane vinasse as substrate. Anaerobe 34:94-105. https://doi.org/10.1016/j.anaerobe.2015.04.008.

29. Zhang T, Liu H, Fang HHP (2003) Biohydrogen production from starch in wastewater under thermophilic condition. J Environ Manage 69:149–156. https://doi.org/10.1016/S0301-4797(03)00141-5.

30. Fernandes BS, Peixoto G, Albrecht FR, Saavedra del Aguila NK, Zaiat M (2010) Potential to produce biohydrogen from various wastewaters. Energy Sustain Dev 14:143-148. https://doi.org/10.1016/j.esd.2010.03.004.

31. Choi J, Ahn Y (2014) Characteristics of biohydrogen fermentation from various substrates. Int J Hydrogen Energy 39:3152-3159. https://doi.org/10.1016/j.ijhydene.2013.12.050.

32. Nigam JN (2000) Cultivation of *Candida langeronii* in sugarcane bagasse hemicellulosic hydrolysate for the production of single cell protein. World J Microbiol Biotechnol 16:367-72. https://doi.org/10.1023/A:1008922806215.

33. Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 34:799–811. https://doi.org/10.1016/j.ijhydene.2008.11.015.

34. Albanez R, Lovato G, Zaiat M, Ratusznei SM, Rodrigues JAD (2016) Optimization, metabolic pathways modeling and scale-up estimative of an AnSBBR applied to biohydrogen production by co-digestion of vinasse and molasses**.** Int J Hydrogen Energy 41:20473-20484. https://doi.org/10.1016/j.ijhydene.2016.08.145.

35. Kiyuna LSM, Fuess LT, Zaiat M (2017) Unraveling the influence of the COD/sulfate ratio on organic matter removal and methane production from the biodigestion of sugarcane vinasse. Bioresour Technol 232:103–112. https://doi.org/10.1016/j.biortech.2017.02.028.

36. Wang CC, Chang CW, Chu CP, Lee DJ, Chang BV, Liao CS, Tay JH (2003) Using filtrate of waste biosolids to effectively produce bio-hydrogen by anaerobic fermentation. Water Res 37:2789–93.https://doi.org/10.1016/S0043-1354(03)00004-6.

37. Guo L, Li XM, Bo X, Yang Q, Zeng GM, Liao DX, Liu JJ (2008) Impacts of sterilization, microwave and ultrasonication pretreatment on hydrogen producing using waste sludge. Bioresour Technol 99:3651-3658. https://doi.org/10.1016/j.biortech.2007.07.026.

38. Mu Y, Yu HQ, Wang G (2007) Evaluation of three methods for enriching H2-producing cultures from anaerobic sludge. Enzym Microb Technol 40:947–953. https://doi.org/10.1016/j.enzmictec.2006.07.033.

39. Rafieenia R, Lavagnolo MC, Pivato A (2018) Pretreatment technologies for dark fermentative hydrogen production: Current advances and future directions. Waste Manag 71:734–748. https://doi.org/10.1016/j.wasman.2017.05.024.

40. Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A, Reid G (2008) The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. Int J Hydrogen Hydrogen Energy 33:4064–4073. http://dx.doi.org/10.1016/j.ijhydene.2008.05.069.

41. Alibardi L, Favaro L, Lavagnolo MC, Basaglia M, Casella S (2012) Effects of heat treatment on microbial communities of granular sludge for biological hydrogen production. Water Sci Technol 66:1483-1490. https://doi.org/10.2166/wst.2012.336**.**

42. Wang J, Wan W (2008) Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. Int J Hydrogen Energy 33:2934– 2941. https://doi.org/10.1016/j.ijhydene.2008.03.048.

43. Khan MA, Ngo HH, Guo W, Chang SW, Nguyen DD, Varjani S, Liu Y, Deng L, Cheng C (2019) Selective production of volatile fatty acids at different pH in an anaerobic membrane bioreactor. Bioresour Technol 283:120–128. https://doi.org/10.1016/j.biortech.2019.03.073.

44. Chen YG, Jiang S, Yuan HY, Zhou Q, Gu GW (2007) Hydrolysis and acidification of waste activated sludge at different pHs. Water Res 41:683–689. https://doi.org/10.1007/s00253-012-4378-4.

45. Chaganti SR, Pendyala B, Lalman JA, Veeravalli SS, Heath DD (2013) Influence of linoleic acid, pH and HRT on anaerobic microbial populations and metabolic shifts in ASBRs during dark hydrogen fermentation of lignocellulosic sugars. Int J Hydrogen Energy 38:2212-2220. https://doi.org/10.1016/j.ijhydene.2012.11.137.

46. Nandi R, Segunpta S (1998) Microbial production of hydrogen: an overview. Crit Rev Microbiol 24:61-84. https://doi.org/10.1080/10408419891294181.

47. Antonopoulou G, Gavala HN Skiadas IV, Angelopoulos K, Lyberatos G (2008) Biofuels generation from sweet sorghum: fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresour Technol 99:110-119. https://doi.org/10.1016/j.biortech.2006.11.048.

48. Morgan-Sagastume F, Pratt S. Karlsson A, Cirne D, P. Lant, Werker A (2011) Production of volatile fatty acids by fermentation of waste activated sludge pre-treated in full-scale thermal hydrolysis plants. Bioresour Technol 102:3089–3097. https://doi.org/10.1016/j.biortech.2010.10.054.

49. Fu Z, Holtzapple MT (2010) Anaerobic mixed-culture fermentation of aqueous ammonia-treated sugarcane bagasse in consolidated bioprocessing. Biotechnol Bioeng 106:216–227. https://doi.org/10.1002/bit.22679.

50. Jiang L, Wang J, Liang S, Wang X, Cen P, Xu Z (2009) Butyric acid fermentation in a fibrous bed bioreactor with immobilized *Clostridium tyrobutyricum* from cane molasses. Bioresour Technol 100:3403–3409. https://doi.org/10.1016/j.biortech.2009.02.032.

51. Etchebehere C, Castelló E, Wenzel J, Anzola-Rojas MPL, Buitrón G, Cabrol L, Carminato VM, Carrillo-Reyes J, Cisneros-Pérez C, Fuentes L, Moreno-Andrade I, Razo-Flores E, Filippi GR, Tapia-Venegas E, Toledo-Alarcón J, Zaiat M (2016) Microbial Communities from 20 Different Hydrogen-Producing Reactors Studied by 454 Pyrosequencing. Appl Microbiol Biotechnol 100:3371-3384. https://doi.org/10.1007/s00253-016-7325-y.

52. Hu C, Giannis A, Chen C, Wang J (2014) Evaluation of hydrogen producing cultures using pretreated food waste. Int J Hydrogen Energy 39:19337–19342. https://doi.org/10.1016/j.ijhydene.2014.06.056.

53. Yang Z, Guo R, Xu X, Wang L, Dai M (2016) Enhanced methane production via repeated batch bioaugmentation pattern of enriched microbial consortia. Bioresour Technol 216:471-477. https://doi.org/10.1016/j.biortech.2016.05.062.

54. Ren NQ, Chua H, Chan SH, Tsang YF, Wang YJ, Sin N (2007) Assessing optimal fermentation type for bio-hydrogen production in continuous-flow acidogenic reactors. Bioresour Technol 98:1774–80. https://doi.org/10.1016/j.biortech.2006.07.026.

55. Li WW, Yu HQ (2011) Physicochemical characteristics of anaerobic H2-producing granular sludge. Bioresour Technol 102:8653–8660. https://doi.org/10.1016/j.biortech.2011.02.110.

56. Wu SY, Hung CH, Lin CN, Chen HW, Lee AS, Chang JS (2006) Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol Bioeng 93:934–946. https://doi.org/10.1002/bit.20800.

57. Hung CH, Cheng CH, Guan DW, Wang ST, Hsu SC, Liang CM, Lin CY (2011) Interactions between *Clostridium* sp. and other facultative anaerobes in a self-formed granular sludge hydrogen-producing bioreactor. Int J Hydrogen Energy 36:8704-8711. https://doi.org/10.1016/j.ijhydene.2010.06.010.

58. Zhao X, Xing D, L. Zhang, Ren N (2010) Characterization and overexpression of a [FeFe]-hydrogenase gene of a novel hydrogen-producing bacterium *Ethanoligenens harbinense.* Int J Hydrogen Energy 35:9598-9602. https://doi.org/10.1016/j.ijhydene.2010.06.098.

59. Castelló E, Braga L, Fuentes L, Etchebehere C (2018) Possible causes for the instability in the H_2 production from cheese whey in a CSTR. Int J Hydrogen Energy 43:2654-2665. https://doi.org/10.1016/j.ijhydene.2017.12.104.

60. Shah AT, Favaro L, Alibardi L, Cagnin L, Sandon A, Cossu R, Casella S, Basaglia M (2016) *Bacillus* sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste. Appl Energy 176:116–124. https://doi.org/10.1016/j.apenergy.2016.05.054.

61. Iyer P, Bruns MA, Zhang HS, Van Ginkel S, Logan BE (2004) H2-producing bacterial communities from a heat-treated soil inoculum. Appl Microbiol Biotechnol 66: 166–173. https://doi.org/10.1007/s00253-004-1666-7.

62. Fuentes L, Braga L, Castelló E, Etchebehere C (2018) Work scheme to isolate the different micro-organisms found in hydrogen-producing reactors: a study of effectiveness by pyrosequencing analysis. J Appl Microbiol 125:96-110. https://doi.org/10.1111/jam.13763.

63. Harun I, Jahim JM, Anuar N, Hassan O (2012) Hydrogen production performance by *Enterobacter cloacae* KBH3 isolated from termite guts. Int J Hydrogen Energy 37: 15052-15061. https://doi.org/10.1016/j.ijhydene.2012.07.101.

64. Sun L, Huang A, Gu W, Ma Y, Zhu D, Wang G (2015) Hydrogen production by *Enterobacter cloacae* isolated from sugar refinery sludge. Int J Hydrogen Energy 40: 1402-1407. https://doi.org/10.1016/j.ijhydene.2014.11.121.

65 Buitrón G, Carvajal C (2010) Biohydrogen production from Tequila vinasses in an anaerobic sequencing batch reactor: effect of initial substrate concentration, temperature and hydraulic retention time. Bioresour Technol 101: 9071-9077. https://doi.org/10.1016/j.biortech.2010.06.127.

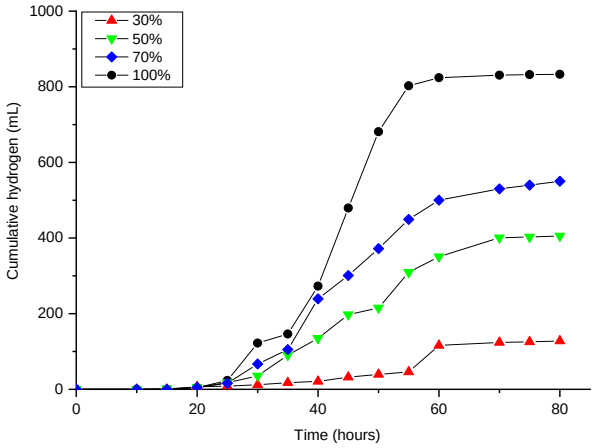

Fig. 1 Cumulative hydrogen production in fermentation assays at different vinasse concentrations at pH6 and T1 pretreatment of the inoculum (90 °C / 10min)

Table 1 Composition of the vinasse used in the experiments

Parameters ($mg.L^{-1}$)	**Initial**
Total calcium	364.80
Total Phosphorus	58.78
Total Magnesium	169.92
Ammonia nitrogen	38.97
Total Nitrogen (Kjeldahl)	394.69
Potassium	2537.65
Sodium	50.47
Sulfides	nd*
Iron	11.73
Manganese	1.67
Chemical Demand Oxygen	20840
Total Suspended Solids	3480
pH	4.22

*nd: not identified by the equipment detection limit

Table 2 Maximum H_2 production rate (Rm), maximum potential of H_2 production (Hmax), lag phase (**λ**), H_2 and VFA yields, substrate consumption (%) and COD removal (%) of vinasse (100%) in fermentation assays performed at different initial pH values and different pretreatments of the inoculum. The values presented are mean of triplicate experiments; the standard deviation of the triplicates is also shown

Pretreatment / Initial pH	Consumed substrate (%)	[1]R_m(mLH_2.h^{-1})	[1]$H_{máx}$ (mL)	[1]λ(h)	[3]H_2 yields	[4]VFA yields	[2]COD removal efficiency (%)
[a]**T1**							
pH 5	39%	80.72 ± 34.56	346.26 ± 53.39	19.12 ± 0.45	2.31	26,88	17%
pH 6	47%	50.39 ± 5.25	821.34 ± 61.92	23.54 ± 0.88	4.75	30,29	25%
pH 7	43%	30.78 ± 10.31	173.75 ± 27.07	13.31 ± 0.13	1.12	44,01	10%
[b]**T2**							
pH 5	40%	49.39 ± 32.01	687.58 ± 60.60	57.03 ± 11.13	4.50	22,81	15%
pH 6	57%	40.38 ± 12.12	376.37 ± 28.66	19.32 ± 0.80	2.78	23,06	10%
pH 7	40%	8.88 ± 1.83	57.67 ± 7.38	23.02 ± 1.25	0.67	16,81	10%
[c]**T3**							
pH 5	28%	13.98 ± 3.17	662.41 ± 32.94	31.71 ± 4.55	4.24	42,62	12%
pH 6	47%	17.66 ± 2.03	697.68 ± 83.07	34.79 ± 0.20	4.47	33,04	12%
pH 7	27%	98.54 ± 17.08	477.89 ± 73.13	17.12 ± 0.01	3.13	79,76	15%

Heat treatment: [a]90°C for 10 min; [a]105°C for 2h; [c]120°C for 20 min (autoclave).
[1]Calculated by adjusting the data to the modified Gompertz model.
[2]COD - Chemical Oxygen Demand.
[3] mmolH_2.g^{1}COD.Considering the influent COD (20.84 mg.L^{-1}- measured at the begin of the experiment).
[4] VFAmg.g^{-1}COD.Considering the influent COD (20.84 mg.L^{-}1- measured at the begin of the experiment).

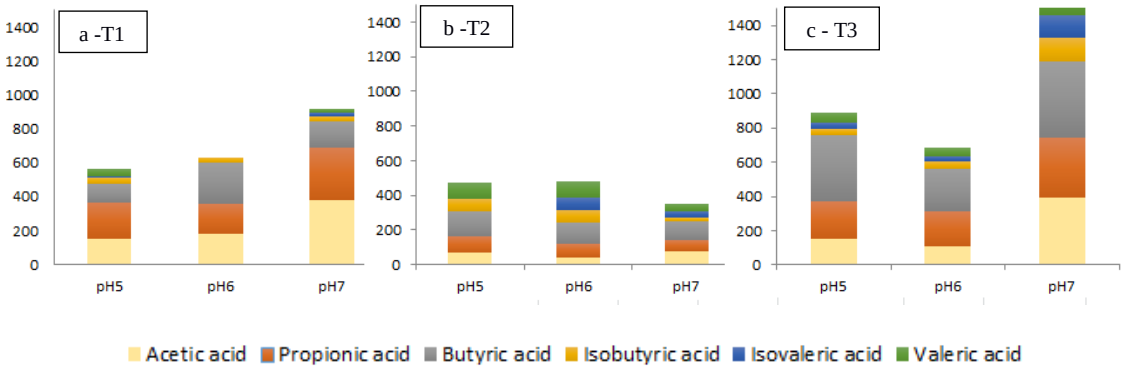

Fig. 2 Production of volatile fatty acids in the assays performed with vinasse at 100% concentration at different initial pH values and distinct pretreatment of the inoculum**.** T1: 90 °C for 10 min; T2: 105 °C for 2 h; T3:120 °C for 20 min (autoclave)

Table 3 Comparison of the hydrogen yield obtained in experiments performed with vinasse as substrate

Inoculum	Substrate	Hydrogen Yield	Reference
Heat treated granular sludge	Sugarcane vinasse	4.75 $mmolH_2g^{1}COD_{influent}$	Present study
Mesophilic consortium	Sugarcane vinasse	2.23 $mmolH_2g^{1}COD_{influent}$	[3]
Anaerobic sludge	Vinasse wastewater	24.97 $mmolH_2g^{-1}COD_{influent}$	[30]
Effluent from a poultry slaughterhouse	Vinasse / molasse (67 -33%)	6.20 $mmolH_2g^{-1}COD_{influent}$	[34]

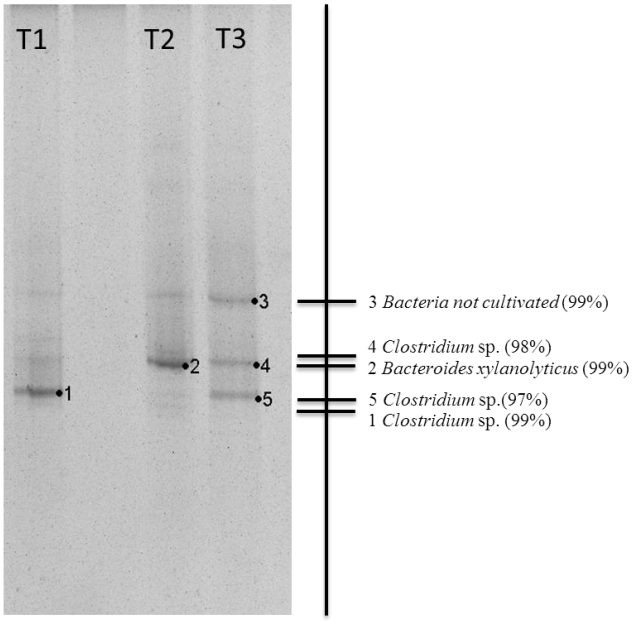

Fig. 3 16S rRNA gene DGGE profiles of the bacterial communities from batch assays using different inocula pretreatment, T1 (pH 6), T2 (pH 5) and T3 (pH6). The DNA from 5 bands were retrieved and the sequences were compared with sequences from databases (NCBI) using BLAST tool, the closest relative and the % of identity according to this sequence analysis was presented next to each DGGE band

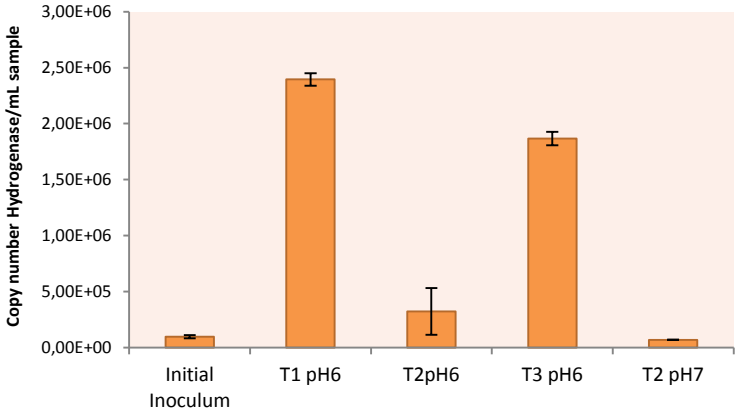

Fig. 4 Fe-hydrogenase copy number in samples taken from the fermentation assays performed at different pH values and different pretreatments of the inoculum. The error bars represent the standard of duplicate measurements

Table 4 Identification of microorganisms and production of hydrogen from the final samples from different fermentation assays (T1, T2 and T3) according to the sequence analysis of 16S rRNA gene

Sample	N° of strain	Similarity	Closer relative	H2 production* ($mLH_2.L^{-1}$)
T1 (pH 6)[a]	3	97%	*Bacillus cereus* (NR 074540)	478.98 ± 29.44
T1 (pH 6)	1	97%	*Enterococcus faecalis* (NR 113901.1)	127.92 ± 125.48
T1 (pH 6)	1	98%	*Bacillus amyloliquefaciens* (NR 117946.1)	393.28 ± 107.09
T1 (pH 6)	1	97%	*Bacillus toyonensis* (NR 121761.1)	572.81 ± 54.13
T1 (pH 6)	2	95%	*Lysinibacillus boronitolerans* (NR 114207.1)	257.79 ± 50.67
T2 (pH 5)[b]	1	98%	*Bacillus subtilis* (NR 102783.2)	1177.74 ± 83.03
T2 (pH 5)	3	99%	*Enterobacter muelleri* (NR 145647.1)	1298.75 ± 276.85
T2 (pH 5)	1	99%	*Enterobacter tabaci* (NR 146667.1)	1155.16 ± 50.23
T3 (pH 6)[c]	6	98%	*Bacillus subtilis* (NR 102783.2)	_
T3 (pH 6)	1	99%	*Enterobacter asburiae* (NR 024640.1)	1013.09 ± 279.48
T3 (pH 6)	1	97%	*Bacillus nakamurai* (NR 151897.1)	315.05 ± 116.59
T3 (pH 6)	1	100%	*Enterobacter muelleri* (NR 145647.1)	_

Heat treatment: [a]90°C for 10min ; [b]105°C for 2h; [c]120°C for 20min (autoclave). * The values presented are mean of triplicate experiments, the standard deviation of the triplicates is also shown.

Supplementary material

Table S1. VFAs yield[a] from 100% vinasse assays with different initial pH values and different inoculum pretreatment: T1: 90 °C for 10 min; T2: 105 °C for 2 h; T3:120 °C for 20 min.

[a]$mg.g^{-1}$COD.Considering the influent COD (measured at the begin of the experiment).

	Acetic acid	Propionic acid	Butyric acid	Isobutyric acid	Isovaleric acid
T1					
pH 5	7.14	10.23	5.40	1.75	0.39
pH 6	8.62	8.49	11.72	1.45	0,00
pH 7	18.17	14.77	7.39	1.37	1.15
T2					
pH 5	3.26	4.67	6.69	3.62	0,00
pH 6	2.03	3.78	5.83	3.63	3.34
pH 7	3.75	3.19	4.98	0.93	1.80
T3					
pH 5	7.22	10.71	18.47	1.88	1.64
pH 6	5.25	9.77	12.03	1.85	1.56
pH 7	18.76	16.86	21.37	6.68	6.21

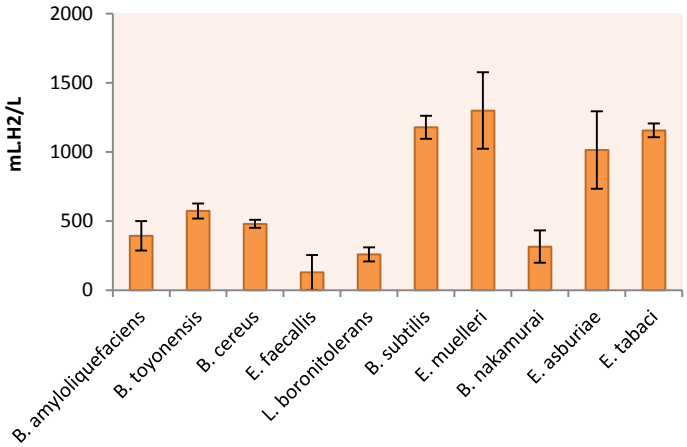

Figure S1: Hydrogen production by isolated microorganisms in the assays performed with vinasse at 50%. The gas samples were taken after 30h of fermentation. The bars indicate the mean value of triplicates, the standard deviation is also shown.

4.2 Capítulo 2

Variation of the prokaryotic and eukaryotic communities after different methods of thermal pretreatment of the inoculum in the hydrogen production from sugarcane vinasse

Flaviane Eva Magrini[1]; Gabriela Machado de Almeida[1]; Denis da Maia Soares[1]; Luiz Gustavo dos Anjos Borges[4]; Leticia Marconatto[4]; Lademir Luiz Beal[2]; Maurício Moura da Silveira[3]; Adriana Giongo[4]; Suelen Paesi[1]

[1]University of Caxias do Sul (UCS), Biotechnology Institute, Molecular Diagnosis Laboratory. E-mail: flavimagrini@hotmail.com; gmalmeida@ucs.br; sopaesi@ucs.br.
[2]University of Caxias do Sul (UCS), Environmental Technologies Laboratory. E-mail: llbeal@ucs.br
[3]University of Caxias do Sul (UCS), Bioprocess Laboratory. E-mail: mmsilvei@ucs.br
[4]Pontifical Catholic University of Rio Grande do Sul (PUCRS), Institute of Petroleum and Natural Resources. E-mail: leticia.marconatto@pucrs.br; luizgaborges@gmail.com; adrianagiongo@gmail.com

Abstract

Hydrogen is a renewable source of energy that can be obtained through microbial consortia or pure cultures that have or have not been pretreated. To understand how inoculum pretreatment and pH affect the diversity of microbial consortia, we propose to compare three methods of thermal pretreatment (P1 = 90 °C/10 min, P2 = 105 °C/120 min and P3= 120 °C/20 min) with different initial pH values (5, 6 and 7) in the production of hydrogen and volatile fatty acids using sugarcane vinasse as substrate. The microbial communities were accessed by high-throughput sequencing using 16S and 18S rRNA genes. The maximum hydrogen production was with the thermal pre-treatment P1 at pH6 (B1) (821.34 mL). The butyric metabolic route was favored in the tests with the three pretreatments. Analysis of the 16S rRNA gene from the microbial communities revealed a similar profile among the bioassays, with a high prevalence of the genus *Clostridium* in the pretreated samples. Firmicutes predominated, being 90% (B1 – P1 pH 6), 95% (B2 – P2 pH 5) and 97% (B3 – P3 pH 6). While in the initial

inoculum (before pretreatments) the predominance was Proteobacteria (31%) and Bacteroidetes (30%). Other genera such as *Bacillus, Syntrophomonas, Geobacter, Syntrophus, Sulfurimonas* were also detected in the higher H_2 production assay. In B3, the highest abundance of *Staphylococcus* (7.9%). The analysis of the 18S rRNA gene for the B1 assay, a high prevalence of *Candida* (47%) and also the presence of Agaricomycetes (5%), Pezizomycotina (5%) and *Aspergillus* (4%). The results have shown the prevalence of *Clostridiaceae* in the three trials (B1, B2, and B3), with the decrease of the microbial diversity with the increase of the temperatures of the pretreatments. The B1 assay had the best performance, as it favored the microorganisms in a consortium to produce H_2 production. The complexity of microbial consortia and possible interaction between bacteria and fungi that act on the degradation of vinasse and the production of hydrogen and volatile fatty acids were observed. The interaction between fungi and bacteria can be explored in future works involving vinasse and other agro-industry residues for the production of bioenergy.

Keywords: microbial community; thermal pretreatment; hydrogen; vinasse; high-performance sequencing

Introduction

Hydrogen is a clean and renewable source and is considered a promising fuel for generating high energy and only water as the final product (Liu, 2008). The bioproduction process of hydrogen can be carried out using several agro-industrial residues as substrate. Among these wastes is vinasse, which is derived from the production of ethanol generated in proportions of 12 to 15 liters per liter of ethanol produced (Sydney et al. 2014). Currently, the vinasse is mainly used as fertilizer for sugarcane cultivation (Salomon & Lora, 2009; Moraes et al., 2014). However, environmental agencies are limiting the amount of vinasse that can be applied to the soil because its pollutant potential is 100 times greater than domestic sewage (Lazaro et al., 2014). Thus, the use of vinasse in anaerobic digestion for the production of hydrogen may be an ecologically correct alternative in the use of this residue.

The biological production of hydrogen can be performed by pure cultures or microbial consortia from inocula from various sources such as soil samples, anaerobic digestion sludge, wastewater from sewage treatment, domestic landfill, among others (Maintinguer et al. 2015). In the environment, the microbial consortia are evolutionarily

associated, acting on substrate degradation and the production of several compounds. Each microorganism present in the microbial consortium plays a specific role in the process, either in the substrate hydrolysis, in the degradation of organic compounds, in the oxygen consumption, or the acidogenesis, hydrogen and acid production, acetogenesis and methanogenesis. Several microorganisms present in microbial consortia are capable of producing hydrogen. Predominantly, there are strictly anaerobic bacteria belonging to the genus *Clostridium*, as *C. butyricum*, *C. pasteurianum*, *C. paraputrificum* e *C. bifermentants* (Sivagurunathan et al., 2014) and, facultative anaerobic microorganisms belonging to the family *Enterobacteriaceae*, as *Enterobacter cloacae* (Maintinguer et al., 2008) and *Klebsiella pneumonae* (Rossi et al., 2011), as well as several species of the genus *Bacillus* (Wang et al., 2010; Motte et al., 2014).

Hydrogen production requires pretreatment of the inoculum to suppress the activity of hydrogen-consuming bacteria, such as methanogens and homoacetogenic that use hydrogen and carbon dioxide to produce methane and acetic acid (Noori & Saady, 2013). The methods of thermal pretreatment of the inoculum have been widely described in the literature (Ren et al. 2008; Kim et al. 2006; Khanal et al. 2004) for the elimination of bacteria that do not form spores, since hydrogen-producing bacteria can form protective spores and survive high pressure, such as the genus *Clostridium*. The thermal pretreatment conditions cited in the literature range from 65 ° C to 121 ° C with a time of exposure between 20 min and 24 h (Ren et al., 2008; Wang & Yin 2017). Several studies (El Bery et al., 2013; Bakonyi et al., 2014; Yang et al., 2017) report the increase in hydrogen production with the thermal pretreatment of the inoculum compared to other pretreatment methods (acid, base, aeration and chloroform) (Wang and Wan, 2008). However, the effect of thermal pretreatment on microbial community composition is still not well known.

The complex composition of the microbial community in the production of hydrogen has been studied by scanning electron microscopy methods, to visualize the structure of the inoculum and, by molecular biology through PCR techniques followed by denaturing gradient gel electrophoresis (PCR-DGGE), real-time PCR (q-PCR), isolation, cloning, sequencing, fluorescence in situ hybridization (FISH), among others (Tolvanen & Karp, 2011). Among them, the high-performance sequencing methods are an alternative, since they have high reliability in identifying the composition of microbial communities (Echebehere et al., 2016). For this reason, this methodology has

been used to characterize microbial consortia of biotechnological interest, such as hydrogen and methane producers (Faust & Raes, 2012).

Yang & Wang (2018) evaluated the composition of the microbial community of fermentative processes for hydrogen production, carried out with inoculums subjected to various pretreatment methods (heat shock, acid, base, aeration and gamma radiation) and observed that abundance of *Clostridium* and *Enterococcus* showed a considerable variation in the different pretreatments. Echebehere et al. (2016) evaluated the microbial community present in 20 reactors used for hydrogen production in four different countries and described remarkably unequal communities with a high predominance of phylum Firmicutes in most samples. In the samples with a high yield of hydrogen, the genera *Clostridium*, *Kosmotoga*, and *Enterobacter* were detected. Already in samples with low hydrogen production, were found *Lactobacillus* and microorganisms of the family *Veillonelaceae*. Knowledge of the microbiota that makes up the fermentation is the key to drive the process and increase the yield of hydrogen production.

Thus, the objective of this study was to compare microbial communities after three methods of thermal pretreatment of the inoculum in the process of hydrogen production in sugarcane vinasse using different initial pH conditions.

Material and methods

Microbial inoculum and pretreatments

The inoculum used was a granular sludge originating from the anaerobic reactor (UASB) used in the vegetable oil processing industry effluent (Pillar, RS, Brazil). The granular inoculum was submitted to three different thermal treatments: 90 °C for 10 min (water bath) (P1) (Kim et al., 2006); 105 °C for 120 min (stove) (P2) (Khanal et al., 2004) and 121 °C for 20 min (autoclave) (P3) (Ren et al., 2008).

Substrate

Pure sugarcane vinasse, without dilution and supplementation, was used as a substrate. The vinasse came from the industry of Guarani Ethanol - Andrade Industrial Unit (Pitangueiras, SP, Brazil) and was kept in the freezer at -20 ° C until processing. Before use, vinasse was centrifuged at 2,000 g for 20 min to the removal of coarse solids, and the supernatant was used in the experiments.

Batch fermentation assays for hydrogen production

The tests were carried out in batch in 600 mL glass bottles containing 300 mL of vinasse and 3 g of inoculum (after the individual thermal pre-treatments). Different pH

values (5, 6 and 7) were evaluated for the production of hydrogen. The initial pH was adjusted using 1M HCl or 1M NaOH solution (APHA, 2005). During the experimental period, the flasks were kept under agitation (Ethik Technology) at 140 rpm at 37 °C and in the absence of light. To guarantee the anaerobiosis, nitrogen gas (N_2) was injected in the liquid medium and the headspace for 10 min. For the isolated microorganisms, the assay was performed in 60 mL glass vials containing 30 mL of pure vinasse culture medium. In this case, the inoculum of each microorganism alone corresponded to the 1 O.D. (optical density) in a spectrophotometer at 600 nm. The bottles were kept as described above. All assays were performed in triplicate.

Chemical analysis

The concentration of the H2 in the biogas was measured by gas chromatography in gas samples taken from the headspace of the batch tests. A chromatographic column CarboxenTM 1006 PLOT Capillary Column (30 m × 0.53 mm) in a Dani Master – Automatic Sample AS chromatographer equipped with a thermic conductivity detector (TCD) was used. Ultra-pure nitrogen gas was used as the carrier gas at 6 mL min-1 flow. The injector temperature was 100ºC, the detector temperature was 230ºC and the column temperature was 40ºC. For the preparation of the calibration curve, volumes of 10, 25, 50, 100, 150, 200 and 250 μL of pure hydrogen gas were injected.

The concentration of volatile fatty acids (acetic, propionic, butyric, isobutyric, valeric and isovaleric acids) and ethanol were determined in liquid samples using a gas chromatograph (GC/MS, Shimadzu - QP2010 Ultra) equipped with a DN - FFAP column (30 m x 0.32 mm x 0.25 μm) with Flame Ionization Detector (FID), with Helium as the carrier gas, as well as synthetic air and nitrogen as auxiliary gases. The column temperature was 100°C for 5 min, increasing by 7°C min-1 to 200°C. The injector and detector temperatures were 200°C and 250°C, respectively. The samples were previously centrifuged (Vision – CE) at 10 min for 10,000 rpm and filtered in a membrane of 0.22 mm.

Chemical oxygen demand (COD) analyzes were performed according to Standart Methods (APHA, 2005). The concentration of total carbohydrates was determined as described by Dubois et al. (1956).

Experimental data fitting

The modified Gompertz equation was used to estimate the kinetic parameters for hydrogen production. The H_2 volume obtained during the tests versus time was treated

with the program Statistica 7 and modeled as Eq. (1), to obtain the kinetic parameters Rm, Hmax and λ.

$$\text{Equation 1: } P = H_{max} \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\}$$

Where, P = cumulative volume of H_2 in the tests, Hmax = maximum volume of H_2 (mL), Rm = maximum rate of H2 production (mL / h), λ = phase lag (h), and t = tests (h).

The volume of hydrogen was converted to mmol by applying the ideal gas equation (PV = nRT), where P is the atmospheric pressure in Caxias do Sul (0.918 atm), V is the volume of H_2 (liters), *n* is the number of moles of H_2, and R is the universal ideal gas constant (0.082 atm L/K.mol), and T is the temperature in the tests (K).

The hydrogen yield was calculated using COD influent (20.84 mg/L, measured at the begin of the experiment) according to the equations: H_2 yield: mmolH_2 / (gCOD x 0.3 L, reaction volume).

Isolation and identification of microorganisms from batch tests

A sample of the final fermentation stage of the tests that demonstrated a better hydrogen production of each of the thermal pretreatments was isolated. For the isolation of the strict anaerobic microorganisms, serial dilutions (1:10) were carried out in PYG medium under anaerobic conditions, and the isolation was performed with the *Roll tube* technique, as described by Castelló et al. (2018). Isolates were characterized using sequence analysis of the 16S ribosomal gene (Poleto et al., 2016). The sequences obtained were deposited in NCBI GenBank with accession numbers MK027301; MK027302; MK027303.

High throughput Sequencing of 16S and 18S rRNA genes

The initial inoculum DNA from the best hydrogen production assays were extracted with the PowerSoil DNeasy Kit (QIAGEN) according to the manufacturer's protocol.

The extracted DNA was amplified by PCR with the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTCTCAT-3') that amplify the V3-V4 region of the 16S rRNA ribosomal gene (Bates et al. 2011). For characterization of eukaryotes (including fungi), fragments of the 18S ribosomal gene were amplified using the primers Fw (5'-ATTAGGGTTCGATTCCGGAGAGG-3') and

Rv (5'-CTGGAATTACCGCGGSTGCTG-3'), described by Nolte et al. (2010). The amplified fragments were analyzed on 1% agarose gel.

PCR products were submitted to high throughput DNA sequencing on the Ion PGM System (Thermo Fisher) following the manufacturer's instructions. The libraries construction was performed with the Ion Plus Fragment Library kit for short amplicons (≤ 350pb), from 100 ng of the amplification product. For the quantification and equalization of the libraries, the Ion Library Equalizer kit was used according to the manufacturer's instructions. An adapter containing unique sequences (IonXpress Barcode kit) was added to each sample so that these could be identified and sequenced in one sequencing. All procedures were performed according to the manufacturer's protocol.

For the PCR reaction emulsion and enrichment steps, the Hi-Q ion PGM view OT2 kit was used with the One Touch system Ion 2. For sequencing, an Ion chip 316 was used with the kit Ion PGM Hi Q View Sequencing according to the manufacturer's instructions. Fragments of the 16S and 18S ribosomal genes generated by the sequencing were submitted to quality control using the PRINSEQ program (Schmieder & Edwards, 2011). The replicate sequences were identified, sorted and filtered to exclude singletons using the USEARCH v8.0.1623 program (Edgar, 2010). The clusters of sequences were assembled using UCLUST algorithm on 97% minimum identity. Chimeras were removed using the RDP reference database (Cole et al., 2013). The taxonomic attribution was obtained using QIIME v1.9 (Caporaso et al., 2010) at 97% of similarity with GreenGenes 13.8 database (De Santis et al., 2006) and Silva111 (Quast et al., 2013) for 16S and 18S rRNA fragments, respectively. The sequences were deposited at the National Center Biotechnology Information (NCBI) in BioProject PRJNA471891.

Microscopy

Images by scanning electron microscopy were obtained in equipment FEI Inspect F50 in secondary electron mode (SE) available at Microscopy and Microanalysis Laboratory Center (LabCEMM) PUCRS. Samples were fixed with 2.5% glutaraldehyde, washed with 0.2M phosphate buffer, post-fixed with 2% osmium tetroxide, dehydrated with acetone and HMDS, and then coated with Au-Pd sputtering.

Additional statistical analysis

Statistical analyses of Shannon and Simpson alpha diversity (observed OTU abundances) and Chao1 (species richness) were performed using the Past3 software

(Hammer et al., 2001). To evaluate the connections between microbial community (8 most abundant genera or OTU) and chemical parameters (substrate consumption (% SC), COD removal (%), hydrogen production (mL), final pH and volatile fatty acids: acetic acid (mg/L), butyric acid (mg/L), propionic acid (mg/L), isovaleric acid (mg/L), valeric acid (mg/L), isobutyric acid (mg/L), the Canonical Correspondence Analysis (ACC) was also performed with the Past3 software.

Results and discussion

Production of hydrogen and volatile fatty acids

Hydrogen production was detected in all assays with vinasse *in natura* and different inoculum pretreatment. Biogas composition analysis showed only hydrogen and CO_2 for all different assays, with no methane, indicating that methanogenesis was avoided under the pretreatment tested. The higher cumulative production of hydrogen was for pretreatment P1 at pH 6 (821 mL), followed by P2 pH 6 (697 mL) and P3 at pH 5 (687 mL) (Fig. 1), with corresponding hydrogen yield of 4.75, 4.5 and 4.47 mmol H_2/gCOD. The lower hydrogen productions (173 and 58 mL) were found at pH 7 in the pre-treatments P1 and P2, respectively. The maximum hydrogen production found in this work 821 mL (P = 29 mmol) was similar to that found by Lazaro et al. (2014) (P = 28.4 mmol) using supplemented vinasse.

The Gompertz model was used to kinetically analyzing the production of hydrogen in the best assays (Table 1). The pretreatments and initial pH distinct resulted in changes in the maximum rate of hydrogen production (R_m) and lag time (**λ)** between the three selected groups. P1 (pH 6) and P2 (pH 5) obtained the highest value of R_m (50.39 $mLH_2.h^{-1}$ and 49.39 $mLH_2.h^{-1}$). In all assays, an extensive lag time (λ) was observed. P1 pH 6 assays obtained the shortest lag time (23.54 h), followed by P3 pH 6 (34.79 h), while P2 at pH 5 obtained the longest lag time (57.03 h) (Table 1). The lag time was directly affected by the initial pH value, but also by the type of substrate used. Vinasse is a complex residue, difficult solubilization and needs a longer time to metabolize by the remaining microorganisms in different pretreatments. This result demonstrated that the different temperatures of the inoculum thermal pretreatments and pH selected different microbial communities, resulting in different efficiencies in hydrogen production.

Fermentation products were evaluated in the samples collected from the fermentation processes with the higher production of hydrogen: P1 at pH 6, P2 at pH 5

and P3 at pH 6, named: B1, B2, and B3, respectively. The main product of the fermentation was butyric acid (HBu), followed by propionic and acetic acid (HAc) mainly in samples B1 and B3, but in B2 samples this difference was lower (Figure 2). At pH 6 the concentration of these acids was higher than at pH 5. However, the higher concentration of isobutyric acid (75.5 mg / L) was found in B2 (pH 5). At pH 7 there was no favoring for hydrogen production in any of the pretreatments.

The results showed that the initial pH had a great effect on the production of hydrogen and favored different metabolic pathways (Figures 1 and 2). The initial pH is a significant factor that affects the fermentative production of hydrogen and the microbial activities, through the influence of the metabolic pathways and the hydrogenase activity. There is no consensus on the optimal pH for hydrogen production since there is a direct relationship with the use of different substrates, fermentation conditions and microorganisms present in the inoculum (Qi et al., 2018).

According to Koskinen et al. (2008), the production of hydrogen from carbohydrates occurs when acetic acid (HAc) or butyric acid (HBu) is produced, whereas if there is the production of ethanol (EtOH) there will be no production of H_2. In these tests, the production of EtOH was not verified. Preferred metabolic pathways for hydrogen production are acetic and butyric (Nandi & Sengupta et al., 1998), which reinforces our results, where the predominant fermentation product of butyric acid was in all three assays. An important factor to evaluate the metabolic production of H_2 is the ratio between the concentrations of butyrate and acetate (HBu / HAc ratio), which may vary with the microbial growth conditions during the fermentation process and has been used to indicate the progress of hydrogen production. The HBu / HAc ratio ranged from 1.35 to 2.28 for indicating a favorable relationship for hydrogen production (Zahedi et al., 2016).

Bacterial community analysis

More than 55,000 16S ribosomal gene fragment sequences were obtained for each sample analyzed. The analysis of the microbial community showed a considerable variation between the initial sample and after the thermal treatments followed the fermentation (Figure 3). In the initial sample, a total of 25 phyla were identified, with a predominance of Proteobacteria (31.2%), Bacteroidetes (30.5%) and 15.7% of the operational taxonomic unit (OTU) Bacteria AC1. These results are similar to those

found by Si et al. (2015), where it was observed a high microbial diversity in this type of sample.

In the samples after fermentation, the predominance was for the Firmicutes phylum with 90.6% (P1), 95.2% (P2) and 97.2% (P3). It was observed that with the increase in temperature in the pretreatments, the predominance of Firmicutes increased, decreasing the microbial diversity in these inoculums (Figure 3). The predominance of the phyla Firmicutes was also observed by other authors in reactors of hydrogen production. Etchebehere et al. (2016) observed the prevalence of this phylum in 25 of the 29 samples of different bioreactors operated in Brazil, Chile, Mexico, and Uruguay. Si et al. (2015), comparing the hydrogen production of granular inoculum in two types of reactors, found 99% of Firmicutes in the UASB (upflow anaerobic reactor) and 91% in the PBR reactor (fixed bed reactor).

In the initial sample, 13 genera and 11 OTUs were identified, showing a considerable microbial diversity in the crude granular inoculum (Figure 4). Comparing the initial inoculum and the samples after fermentation, it is possible to observe a variation of the microbial diversity before and after the pretreatments. The microorganisms present at the beginning of fermentation (Figure 4) are fundamental in the process, since some genera such as *Syntrophomonas*, *Bacillus*, *Mycoplana* and *Chryseobacterium* act in the hydrolysis process of the organic matter transforming it into simpler compounds for bacterial assimilation (Gan et al., 2012; Shah et al., 2016; Gao et al., 2017; Wang et al., 2016).

In the tests B1, B2 and B3, there is a reduction of microbial diversity and a high predominance of the genus *Clostridium* and an OTU belonging to *Clostridiaceae* family, with relative abundances comprising 90% for each pretreatment (Figure 4). It indicates that the three pretreatments favor these hydrogen-producing microorganisms. *Clostridium* is a bacterium described as a producer of hydrogen due to its high growth rate, high yields in the process and the capacity to use several carbon sources as a substrate (Lin et al., 2007). Different *Clostridium* species have already been used in fermentative trials for the production of hydrogen, such as *Clostridium beijerinckii*, *Clostridium butyrene*, *Clostridium tyrobutyricum*, *Clostridium acetobutylicum* and *Clostridium saccharoperbutylacetonicum* (Abdeshahian et al., 2014).

However, some *Clostridium* species also act as homoacetogenic bacteria, consume hydrogen to produce acetic acid (Saady 2013), or even produce lactate and propionate by lowering hydrogen yield (Hawkes et al., 2007). A significant abundance

of the genus *Clostridium* was detected in the higher hydrogen production (B1) assay, but the 16S ribosomal gene sequences obtained in the high-performance sequencing were too short (about 250 nucleotides), which did not allow to classify sequences at the species level (Fig. 3).

In B3, the greatest abundance of the genus *Staphylococcus* (7.9%) (Figure 4 and Table 2) was obtained, which besides acting on the oxygen consumption, also produces extracellular polymeric substances (EPS), which act to form the structures (Hung et al., 2011) playing a vital role in the integrity of the granules. Possibly the high temperature and the pressure of this pretreatment favored the aggregation of *Staphylococcus* species and consequently the production of EPS, which conferred resistance and stability of the granules in this assay.

Other genera such as *Bacillus*, *Syntrophomonas*, *Geobacter*, *Syntrophus*, *Sulfurimonas* were also detected mainly in B1 and the initial inoculum (Figure 4 and Table 2) and unidentified in B2 and B3. These genera have an essential role in the microbial consortium, either in the substrate hydrolysis or the degradation of volatile fatty acids, or even in the case of *Sulfurimonas*, they act in the sulfur oxidation in sulfate (Shah et al., 2012; Gan et al., 2012, Zhang et al., 2015).

In addition to the microorganisms mentioned above, other genera were also found in relative abundances smaller than 1% of total sequences for B1, B2, and B3, but higher in the initial inoculum (Table 2). *Stenotrophomonas* from the Proteobacteria phylum, act in the process of acetogenesis (Dhar et al., 2015). *Arcobacter, Mycoplana, Chryseobacterium,* and *Flavobacterium* assist in substrate hydrolysis, degradation of amino acids and aromatic compounds (Ruiz et al., 2014, Gao et al., 2017; Wang et al., 2016), are strict halotolerant and thiosulfate-reducing anaerobes (Li et al., 2008). Already, *Fusibacter* genus is a strict anaerobe halotolerant and, reducing thiosulfate (Li et al., 2008).

To measure the microbial diversity, the Shannon (H'), Simpson (D) and Chao 1 indices were applied, indicating similar diversity (71) for the microorganisms from the B2 and B3 assays, with a decrease in diversity in relation to B1 (100) and compared to the initial inoculum (Table 3). The canonical correspondence analysis (CCA) (Fig. 5) shows that there is a correlation between hydrogen production (B1) with the order Bacteroidales and SHA-114 OTUs. The hydrogen production (B1) was also correlated with the high initial acetic acid concentration and abundances of Geobacter. Currently, this genus has been widely described in anaerobic digestion of waste due to its ability to

transfer extracellular electrons (Mei et al. 2018). About 40% of the Geobacteraceae family is able to use hydrogen as an electron donor (Coppi et al. 2004). Further, Geobacter is capable of oxidizing acetate, which might explain the consumption of acetic acid during the assays B1 (Figure 2). This genus is efficient for generating electric current and producing bioelectrochemical hydrogen through microbial electrolysis cells (MECs), improving hydrogen yield (HY) (Wang et al. 2018), which may be promising in the future work to improve efficiency in the production of hydrogen from sugarcane vinasse.

The fermentation process in B3 is related to the great abundance of *Staphylococcus*, which acting in the production of EPS favoring the anaerobic environment due to the maintenance of the granule and, may also be involved in the production of hydrogen, as described in previous work (Poleto et al., 2016). B3 is also related to the presence of isovaleric acid that can be oxidized to form hydrogen, acetic acid, and propionic acid, which demonstrated a higher amount in this pretreatment while B2 is correlated to the production of valeric and isobutyric acids. *Clostridium* and a OTU belonging to the *Clostridiaceae* family are related to the three assays.

Six strains of strictly anaerobic microorganisms from the assay B1, four strains from the B2 and B3 assays were isolated. Of these isolates, three species were identified: *Clostridium bifermentans*, *C. saccharoperbutylacetonicum* and *C. saccharobutylicum* (Table 4). The species of *C. bifermentans* and *C. saccharoperbutylacetonicum* are already reported as producers of biohydrogen (Alalayah et al., 2008; Al-Shorgani et al., 2014; Wong et al., 2018). *C. saccharobutylicum* is cited in the literature as a producer of butanol and 1,3-propanediol (Gungormusler et al., 2010; Ujor et al., 2015). *Clostridium* species are very versatile because they can utilize a variety of carbohydrate sources, including cellulosic, hemicellulosic and starch materials (Al-Shorgani et al., 2011). Thus, they are promising candidates in the bioproduction of compounds of biotechnological interest.

These isolates were evaluated for hydrogen production alone and associated with co-cultures (Table 4). *C. bifermentans* alone was the best producer of hydrogen in vinasse (435 mL H_2). When associated with co-culture *C. bifermentans* and *C. saccharoperbutylacetonicum*, the hydrogen production increased to 529 mL H_2. It corroborated with other studies describing the potential of *C. bifermentans* in the

production of hydrogen from vinasse (Rabelo et al., 2019), glucose (Singh et al., 2010) or wastewater sludge (Wang et al., 2003). The other *Clostridium* species had a low hydrogen production in both, alone and in co-cultures, using vinasse as substrate (Table 3).

Analysis of the fungal community

Both the sludge and vinasse used in experiments are environmental samples with complex microbiota, so in addition to the bacterial community described above, the fungal community was also evaluated in the fermentation trials. The fungi were evaluated in the initial inoculum and after fermentation of the B1 assay (Table 5). The unicellular fungus *Candida* was found in great abundance in the initial inoculum (24%) and increased 50% to the final process (47%). Possibly the considerable increase of *Candida* can be due to the composition of the vinasse used as a substrate, which favored the growth of this microorganism, being a product of the sugar and alcohol industry. However, the presence of *Candida* in the microbial community may be an inhibiting factor in the production of biohydrogen during acidogenesis due to the production of antimicrobial compounds (usually proteins or low molecular weight glycoproteins <20 kDa) (Detman et al., 2018). In future works can be evaluated efficient methods of pretreatment in the control of the yeast population in the tests of hydrogen production.

In the sample after fermentation, they were also found in significant proportions class Agaricomycetes (5.2%) and Pezizomycotina subphylum (4.6%), which are common fungi in activated sludge (Wang et al., 2018). They play an essential role in the fermentation process since they synthesize enzymes that digest polysaccharides (Zhao et al., 2013), aiding the hydrolysis of the substrate, facilitating the absorption of hydrolytic bacteria into hydrogen and volatile fatty acids.

The filamentous fungus *Aspergillus* remained constant from start to end of fermentation (~ 4%). Some species of *Aspergillus* are sources of enzymes such as pectinases, proteases, and amyloglucosidases, which may aid in the hydrolysis process, forming a hydrolyzate rich in nutrients and facilitating the production of hydrogen (Han et al., 2015).

The presence of fungi in the fermentation process is due to the fact that they are more resistant than bacteria, due to their persistence characteristics in environments with a large amount of organic matter (Wang et al., 2018). However, Hernández et al. (2019), noted that the loss greater diversity was to fungi than to bacteria after heat

treatment of the inoculum (105 °C for 24 hours) and, bacterial communities recover more readily than fungal communities.

Some works suggest the bioaugmentation of activated sludge with fungi capable of degrading pollutants, mainly phenols or fungi specialized in degrading lignocellulosic substrates, capable of converting polysaccharides into simple sugars that can be subsequently fermented, improving the efficiency for subsequent hydrogen production (Wang et al., 2018; Nkemka et al., 2016). This hydrolysis process requires the synergistic action of *pools* of enzymes that in many cases, only mixed cultures composed of fungi and bacteria can produce (Hernández et al., 2019).

Microbial morphology in the different assays

In the scanning electron microscopy (SEM) images of the initial inoculum (a) before the pretreatments, an agglomerate of several microorganisms forming the granular structure of the sludge is observed (Figure 6a).

The samples after the thermal treatments B1 (Figure 6b) and B2 (Figure 6c) were very similar morphologically, with the predominance of bacilli-like structures, possibly bacteria of the genus *Clostridium*, which was found in high abundance in the sequencing analysis. While in the B3 sample (Figure 6d), the inoculum presents with a higher amount of bacilli and cocci, possibly *Staphylococcus*, that produce EPS and for this reason, the inoculum is more compacted than in the other pre-treatments.

In this study, the B1 assay had the best performance in the hydrogen production of the others (B2 and B3), indicating that under these conditions the strict anaerobic microorganisms (*Clostridium*) responsible for the efficiency of hydrogen production were favored, as well as the facultative anaerobic microorganisms (bacteria and fungi), which are extremely important in the maintenance of anaerobic and substrate hydrolysis.

Not only composition and microbial diversity are important, but also the abundance and interaction between the different microorganisms that drive the process. Also, we observed that *Clostridium* alone or associated with other species of this genus (Table 3) do not perform the same performance as when related to other facultative bacteria as observed in the microbial consortium, which indicates once again that the interaction with other microorganisms is fundamental for fermentation success.

The production of hydrogen via microbial consortium is already described as a complicated process, and this is increased when associated with agro-industrial

residues, such as sugarcane vinasse. Besides the bacterial diversity of the inoculum and the substrate, we have the presence of fungi, which are also part of this microecosystems and play a key role in microbial ecology, assisting in the process of degradation and hydrolysis of vinasse used as substrate.

Conclusion

Thermal pretreatments and initial pH values showed an influence on the composition of the microbial community and consequently on hydrogen production efficiency, as well as on the composition and concentration of volatile fatty acids.

B1 (90 °C / 10min at pH 6) showed a better hydrogen production than the others (B2 and B3), with a maximum production (Hmax) of 821.34 mL H_2. In all three assays (B1, B2, and B3) the metabolic butyric route was predominant. However, at pH 6 the concentration of these acids was higher than in the pH 5 assay.

With the increase in temperature of the pretreatments, there was also an increase in the predominance of the Firmicutes phyla, with 90.6% (B1), 95.2% (B2) and 97.2% (B3) and consequently decreased microbial diversity.

In B1, where there was a higher production of hydrogen, there was a great abundance of the genus *Clostridium* and the presence of *Bacillus*, *Syntrophomonas*, *Geobacter*, *Arcobacter*, *Stenotrophomonas*, besides the correlation with Bacteroidales. In addition to the fungi Agaricomycetes, Pezizomycotina, and *Aspergillus* described as necessary in the hydrolytic process of vinasse.

The results obtained in this work show the influence of the pretreatments on the microbiota in the vinasse bioconversion for the production of hydrogen, valuing this residue of the ethanol production generated in large quantities daily, reducing its environmental impact.

Acknowledgment

The authors thank Petrobras for their financial support, the University of Caxias do Sul and the Pontifical Catholic University of Rio Grande do Sul, in particular, the Institute of Petroleum and Natural Resources (IPR/PUCRS) and the Laboratory of High Performance (LAD/PUCRS). Luiz Gustavo dos A. Borges thanks PEGA/PUCRS.

References

Abdeshahian, P.; Al-Shorgani, N.K.N.; Salih, N.K.M.; Shukor, H.; Kadier, A.; Hamid, A.A.; Kalil, M. S. (2014). The production of biohydrogen by a novel strain Clostridium sp. YM1 in dark fermentation process. International Journal of Hydrogen Energy, 39: 12524-12531.

Alalayah WM, Kalil MS, Kadhum AAH, Jahim JM, Alauj NM (2008) Hydrogen production using Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564). Int J Hydrog Energy 33(24): 7392–7396.

Albanez, R.; Lovato, G.; Zaiat, M.; Ratusznei, S.M.; Rodrigues, J.A.D. (2016). Optimization, metabolic pathways modeling and scale-up estimative of an AnSBBR applied to biohydrogen production by co-digestion of vinasse and molasses. International Journal of Hydrogen Energy, 41: 20473-20484.

Al-Shorgani NKN, Kalil MS, Yusoff WMW (2011) The effect of different carbon sources on biobutanol production using Clostridium saccharoperbutylacetonicum N1-4. Biotechnology 10:280–285. doi:10.3923/biotech.2011.280.285

Al-Shorgani, N.K.N.; Tibin, E.M.; Ali, E.; Hamid, A.A.; Yusoff, W.M.W.; Kalil, M.S. (2014). Biohydrogen production from agroindustrial wastes via Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564). Clean Techn Environ Policy, 16:11–21.

APHA, 2005. Standart methods for the examination of water and wastewater. 20th edition. Washington: American Public Health Association.

Baek, G.; Kim, J.; Cho, K.; Bae, H.; Lee, C. (2015). The biostimulation of anaerobic digestion with (semi)conductive ferric oxides: their potential for enhanced biomethanation. Appl. Microbiol. Biotechnol, 99: 10355–10366.

Bakonyi P, Borza B, Orlovits K, Simon V, Nemestothy N, Belafi-Bako K. (2014).Fermentative hydrogen production by conventionally and unconventionally heat pretreated seed cultures: a comparative assessment. Int J Hydrogen Energy, 39:5589-5596. https://doi.org/10.1016/j.ijhydene.2014.01.110.

Bates S. T., Berg-Lyons D., Caporaso J. G., Walters W. A.(2011). Knight R., Fierer N. Examining the global distribution of dominant archaeal populations in soil. The ISME journal, 5: 908-917.
https://doi.org/10.1038/ismej.2010.171

Caporaso J. G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F. D., Costello E. K., Fierer N., Peña A.G., Goodrich J.K., Gordon J. I., Huttley G. A., Kelley S.T., Knights D., Koenig J.E., Ley R.E., Lozupone C.A., McDonald D., Muegge B.D., Pirrung M ., Reeder J., Sevinsky J.R., Turnbaugh P.J., Walters W.A., Widmann J., Yatsunenko T., Zaneveld J., Knight R.. (2010). QIIME allows analysis of high-throughput community sequencing data. Nature Methods, 7335-336.

Cole J. R., Wang Q., Fish J. A., Chai B., Mcgarrell D. M., Sun Y., Brown C.T., Porras-Alfaro A., Kuske C.R., Tiedje J. (2013). Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res., 4:633-642 https://doi.org/10.1093/nar/gkt1244.

Coppi, M.V., O'Neil, R.A., Lovley, D.R. (2004). Identification of an Uptake Hydrogenase Required for Hydrogen-Dependent Reduction of Fe (III) and Other Electron Acceptors by *Geobacter sulfurreducens.* Journal of bacteriology, 186:3022–3028 DOI: 10.1128/JB.186.10.3022–3028.2004.

Detman, A., Chojnacka, A., Mielecki, D., Błaszczyk, M.K., Sikora, A. (2018). Inhibition of hydrogen-yielding dark fermentation by ascomycetous yeasts. International Journal of Hydrogen Energy, 43:10967-10979.

Dhar, B.R.; Elbeshbishy, E.; Hafez, H.; Lee, H. (2015). Hydrogen production from sugar beet juice using an integrated biohydrogen process of dark fermentation and microbial electrolysis cell. Bioresource Technology, 198: 223–230.

Etchebehere, C.; Castelló, E.; Wenzel, J.; Anzola-Rojas, M.P.; Borzacconi, L.; Buitrón, G.; Cabrol, L.; Carminato, V.M.; Carrillo-Reyes , J.; Cisneros-Pérez , C.; Fuentes, L.; Moreno-Andrade, I.; Razo-Flores, E.; Filippi, G.R.; Tapia-Venegas, E.; Toledo-Alarcón, J.; Zaiat, M. (2016). Microbial communities from 20 different hydrogen-producing reactors studied by 454 pyrosequencing. Appl Microbiol Biotechnol, 100:3371–3384.

Edgar R. C. Search and clustering orders of magnitude faster than BLAST. (2010). Bioinformatics 26 2460-2461.

Faust, K.; Raes, J. (2012). Microbial interactions: from networks to models. Nature Reviews Microbiology, 10: 538-550.

Gan, Y., Qiu, Q., Liu, P., Rui, J., Lu, Y. (2012). Syntrophic oxidation of propionate in rice field soil at 15 and 30C under methanogenic conditions. Appl. Environ. Microbiol, 78: 4923–4932.

Gao, Y.; Wang, C.; Zhang, W.; Di, P.; Yi, N.; Chen, C. (2017). Vertical and horizontal assemblage patterns of bacterial communities in a eutrophic river receiving domestic wastewater in southeast China. Environmental Pollution, 230: 469-478.

Gungormusler, M.; Gonen, C.; Ozdemir, G.; Azbar, N. (2010). 1,3-Propanediol production potential of Clostridium saccharobutylicum NRRL B-643. New Biotechnology, 27:6.

Hammer O, Harper DAT, Ryan PD. (2001). Past: paleontological statistics software sackage for education and data analysis. Palaeontol Electron 4:1–9.

Han, W., Ye, M., Zhu, A.J., Zhao, H.T., Li, Y.F. (2015). Batch dark fermentation from enzymatic hydrolyzed food waste for hydrogen production. Bioresource Technology, 191:24–29.

Hawkes, F.R.; Hussy, I.; Kyazze, G.; Dinsdale, R.; Hawkes, D.L. (2007). Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. International Journal of Hydrogen Energy, 32: 172–184.

Hung, C.H.; Chang, Y.T.; Chang, Y.J. (2011). Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems – A review. Bioresource Technology, 102: 8437–8444.

Khanal, S.K. Chen, W.H.; Li, L.; Sung, S. (2004). Biological hydrogen production: effects of pH and intermediate products. International Journal of Hydrogen Energy 29 (2004) 1123 – 1131.

Kato, S.; Hashimoto, K.; Watanabe, K. (2012). Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. Environ. Microbiol. 14:1646–1654.

Kim, D.H.; Han, S.K.; Kim.; S.H.; Shin, H.S. (2006). Effect of gas sparging on continuous fermentative hydrogen production. International Journal of Hydrogen Energy, 31:2158-2169.

Kim, D.H.; Han, S.K.; Kim.; S.H.; Shin, H.S. (2006). Effect of gas sparging on continuous fermentative hydrogen production. International Journal of Hydrogen Energy, 31:2158-2169.

Koskinen, P.E.P.; Beck, S.R., J.; Orlygsson, Puhakka, L.A. (2008). Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from icelandic geothermal areas. Biotechnol. Bioeng., 101: 679-690.

Kuroda, K.; Nobu, M.K.; Mei, R.; Narihiro, T.; Bocher, B.T.W.; Yamaguchi, T.; Liu, W.T. (2016) A single- granule-level approach reveals ecological heterogeneity in an upflow anaerobic sludge blanket reactor. PLoS ONE 11(12): e0167788. doi:10.1371/journal.pone.0167788.

Lazaro, C. Z.; Perna, V.; Etchebehere, C.; Varesche, M. B. A. (2014). Sugarcane vinasse as substrate for fermentative hydrogen production: The effects of temperature and substrate concentration. International Journal of Hydrogen Energy, 39: 6407-6418.

Li, D.; Hendry, P.; Faiz, M. (2008). A survey of the microbial populations in some Australian coalbed methane reservoirs. International Journal of Coal Geology 76:14–24.

Liu, D. (2008). Bio-hydrogen production by dark fermentation from organic wastes and residues. PhD Thesis. Department of Environmental Engineering Technical, University of Denmark, Denmark.

Maintinguer, S.I.; Fernandes, B.S.; Duart, I.C.C.; Saavedra, N.K.; Adorno, M.A.T.; Varesche, M.B. (2008). Fermentative hydrogen production by microbial consortium. International Journal of Hydrogen Energy, 33:4309-4317.

Maintinguer, S.I.; Sakamoto, I.K.; T. Adorno, M.A.; A. Varesche, M.B. (2015). Bacterial diversity from environmental sample applied to bio-hydrogen production. International Journal of Hydrogen Energy, 40: 3180-3190.

McInerney, M.J., Rohlin, L., Mouttaki, H., Kim, U., Krupp, R.S., Rios-Hernandez, L., Sieber, J., Struchtemeyer, C.G., Bhattacharyya, A., Campbell, J.W., Gunsalus, R.P. (2007). The

genome of Syntrophus aciditrophicus: life at the thermodynamic limit of microbial growth. Proc. Natl. Acad. Sci. 104:7600–7605.

Mei, R., Nobu, M.K., Narihiro, T., Yu, J., Sathyagal, A., Willman, E., Liu, W.T. (2018). Novel Geobacter species and diverse methanogens contribute to enhanced methane production in media-added methanogenic reactors. Water Research, 147:403-412.

Moraes, B.S.; Junqueira, T.L.; Pavanello, L.G.; Cavalett, O.; Mantelatto, P.E.; Bonomi, A.; Zaiat, M. (2014). Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? Applied Energy, 113:825–835.

Motte, J.C.; Trably, Eric.; Hamelin, J.; Escudié, R.; Bonnafous, A.; Steyer, J.P.; Bernet, N.; Delgenès, J.P.; Dumas, C. (2014). Total solid content drives hydrogen production through microbial selection during thermophilic fermentation. Bioresource Technology, 166:610–615.

Nandi, R., Segunpta, S. (1998). Microbial production of hydrogen: an overview. Crit Rev Microbiol, 24:61-84.

Nkemka, N.V.; Gilroyed, B., Yanke, J., Gruninger, R., Vedres, D., McAllister, T., Hao, X. (2015). Bioaugmentation with an anaerobic fungus in a two-stage process forbiohydrogen and biogas production using corn silage and cattail. Bioresource Technology, 185: 79-88.

Nolte, V., R. V. Pandey, S. Jost, R. Medinger, B. Ottenwalder, J. Boenigk, and C. Schlotterer. 2010. 'Contrasting seasonal niche separation between rare and abundant taxa conceals the extent of protist diversity', Mol Ecol, 19: 2908-15.

Noori, M.; Saady, C. (2013). Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unresolved challenge. International Journal of Hydrogen Energy, 38:13172–13191.

Poleto, L.; Souza, P.; Magrini, F.E.; Beal, L.L.; Torres, A.P.R.; Sousa, M.P.; Laurino, J.P.; Paesi, S. (2016). Selection and identification of microorganisms present in the treatment of wastewater and activated sludge to produce biohydrogen from glycerol. International journal of hydrogen energy 41:-4374-4381.

Qi, N., Hu, X., Zhao, X., Li, L., Yang, J., Zhao, Y., Li, X. (2018). Fermentative hydrogen production with peanut shell assupplementary substrate: Effects of initial substrate, pH andinoculation proportion. Renewable Energy, 127:548-558.

Rabelo, C.A.B.S.; Soares, L.A.; Sakamoto, I.K.; Silva, E.L.; Varesche, M.B.A. (2018). Optimization of hydrogen and organic acids productions with autochthonous and allochthonous bacteria from sugarcane bagasse in batch reactors. Journal of Environmental Management, 223: 952–963.

Ren, N.Q.; Guo, W.Q.; Wang, X.J.; Xiang, W.S.; Liu, B.F.; Wang, X.Z.; Ding, J.; Chen, Z.B. (2008). Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. International Journal of Hydrogen Energy, 33: 4318 – 4324.

Rossi, D.M.; Costa, J.B.; Souza, E.A.; Peralba, M.C.R.; Samios, D.; Ayub, M.A.Z. (2011). Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. International Journal of Hydrogen Energy, 36:4814-4819.

Ruiz, V.; Ilhan, Z.; Kang, D; Krajmalnik-Brownb, R.; Buitrón, G. (2014). The source of inoculum plays a defining role in the development of MEC microbial consortia fed with acetic and propionic acid mixtures. Journal of Biotechnology, 182–183: 11–18.

Salomon, K. R.; Lora, E. E. S. (2009). Estimate of the electric energy generating potential for different sources of biogas in Brazil. Biomass and bioenergy, 33: 1101–1107.

Shah, A.T.; Favaro, L.; Alibardi, L.; Cagnin, L.; Sandon, A.; Cossu, R.; Casella, S.; Basaglia, M. (2016). Bacillus sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste. Applied Energy 176 (2016) 116–124.

Schmieder, R., Edwards R. (2011). Quality control and preprocessing of metagenomic datasets. Bioinformatics,27:863-864. https://doi.org/10.1093/bioinformatics/btr026.

Si, B, Liu Z, Zhang, Y., Li, J., Xing, X-H, Li, B., Duan, N., Lu, H. (2015). Effect of reaction mode on biohydrogen production and its microbial diversity. Int J Hydrog Energy, 40:3191–3200.

Si, B.; Liu, Z.; Zhang, Y.; Li, J.; Shen, R.; Zhu, Z.; Xing, X. (2016). Towards biohythane production from biomass: Influence of operational stage on anaerobic fermentation and microbial community. international journal of hydrogen energy, 41: 4429-4438.

Singh, S.; Sudhakaran, A.K.; Sarma, P.M.; Subudhi, S.; Mandal, A.K.; Gandham, G.; Lal, B. (2010). Dark fermentative biohydrogen production by mesophilic bacterial consortia isolated from riverbed sediments. Int J Hydrog Energy, 35: 10645–10652.

Sivagurunathan, P.; Sen, B.; Lin, C.Y. (2014). Batch fermentative hydrogen production by enriched mixed culture: Combination strategy and their microbial composition. Journal of Bioscience and Bioengineering, 117:222-228.

Sydney, E.B.; Larroche, C.; Novak, A.C.; Nouaille, R.; Sarma, S.J.; Brar, S.K.; Letti, L.A.J.; Soccol, V. T.; Soccol, C.R. (2014). Economic process to produce biohydrogen and volatile fatty acids by a mixed culture using vinasse from sugarcane ethanol industry as nutrient source. Bioresource Technology, 159:380-386.

Slezak, R.; Grzelak, J.; Krzystek, L.; Ledakowicz, S. (2017). The effect of initial organic load of the kitchen waste on the production of VFA and H2 in dark fermentation. Waste Management (2017), http://dx.doi.org/10.1016/j.wasman.2017.06.024.

Shu, D.; He, Y.; Yue, H.; Wang, Q. (2015). Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high-throughput pyrosequencing. Bioresource Technology, 186:163–172.

Tolvanen, K, Karp, M.T. (2011) Molecular methods for characterizing mixed microbial communities in hydrogen-fermenting systems. Int J Hydrog Energy, 36:5280–5288.

Tsitko, I; Lusa, M.; Lehto, J.; Parviainen, L.; Ikonen, A.T.K.; Lahdenperä, A.-M.; Bomberg, M. (2014) The Variation of Microbial Communities in a Depth Profile of an Acidic, Nutrient-Poor Boreal Bog in Southwestern Finland. Open Journal of Ecology, 4: 832-859. http://dx.doi.org/10.4236/oje.2014.413071.

Ujor, V.; Bharathidasan, A.K., Michel Jr., F.C.; Ezeji, T.C.; Cornish, K. (2015). Butanol production from inulin-rich chicory and Taraxacumkok-saghyz extracts: Determination of sugar utilization profile ofClostridium saccharobutylicum P262. Industrial Crops and Products, 76:739–748.

Wang, A.; Gao, L.; Ren, N.; Xu, J.; Liu, C.; Lee, D.J. (2010). Enrichment strategy to select functional consortium from mixed cultures: Consortium from rumen liquor for simultaneous cellulose degradation and hydrogen production. International Journal of Hydrogen Energy, 35:13413–13418.

Wang, C.C.; Chang, C.W.; Chu, C.P.; Lee, D.J.; Chang, B.V.; Liao, C.S. (2003). Producing hydrogen from wastewater sludge by Clostridium bifermentans. Journal of Biotechnology 102: 83-92.

Wang J, Yin Y. (2017). Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. Int J Hydrogen Energy, 42:4804-4823.

Wang, J.; Wan, W. (2008). Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. International Journal of Hydrogen Energy, 33:2934 – 2941.

Wang, L., Singh, L., Liu, H. (2018). Revealing the impact of hydrogen productionconsumption loop against efficient hydrogen recovery in single chamber microbial electrolysis cells (MECs). International Journal of Hydrogen Energy, 43:13064:13071.

Wang, X.; Wang, Y.; Zhang., Duo, C., Zhao, C., Xie, F. (2018). Isolation of a highly efficient phenol-degrading fungus and the preparation of an effective microbial inoculum for activated sludge and its enhancement for hydrogen production, International Journal of Hydrogen Energy, https://doi.org/10.1016/j.ijhydene.2018.10.154.

Wang, Y.; Wang, Q.; Li , M.; Yang, Y.; He, W.; Yan, G.; Guo, S. (2016). An alternative anaerobic treatment process for treatment of heavy oil refinery wastewater containing polar organics. Biochemical Engineering Journal, 105:44–51.

Wong, Y.M.; Wu, T.Y.; Ling, T.C.; Show, P.L.; Lee, S.Y.; Chang, J.S.; Ibrahim, S.; Juan, J.C. (2018). Evaluating new bio-hydrogen producers: Clostridium perfringens strain JJC, Clostridium bifermentans strain WYM and Clostridium sp. strain Ade.TY. Journal of Bioscience and Bioengineering, 125:590-598.

Yang, G.; Wang, J. (2018). Kinetics and microbial community analysis for hydrogen production using raw grass inoculated with different pretreated mixed culture. Bioresource Technology, 247: 954–962.

Yang, Z.; Guo, R.; Xu, X.; Wang, L.; Dai, M. (2016). Enhanced methane production via repeated batch bioaugmentation pattern of enriched microbial consortia. Bioresource Technology, 2016:471-477.

Yang, S.; Phan, H.; Bustamante, H.; Guo, W.; Ngo, H.; Nghiem, L. (2017). Effects of shearing on biogas production and microbial community structure during anaerobic digestion with recuperative thickening. Bioresource Technology, 234:439–447.

Zahedi, S.; Solera, R.; García-Morales, J. L.; Sales, D. (2016). Effect of the addition of glycerol on hydrogen production from industrial municipal solid waste. Fuel. 180: 343-347.

Zhao, L., Cao, G.L., Wang, A.J., Guo, W.Q., Ren, H.Y., Ren, N.Qi. (2013). Simultaneous saccharification and fermentation of fungal pretreated cornstalk for hydrogen production using Thermoanaerobacterium thermosaccharolyticum W16. Bioresource Technology, 145:103–107

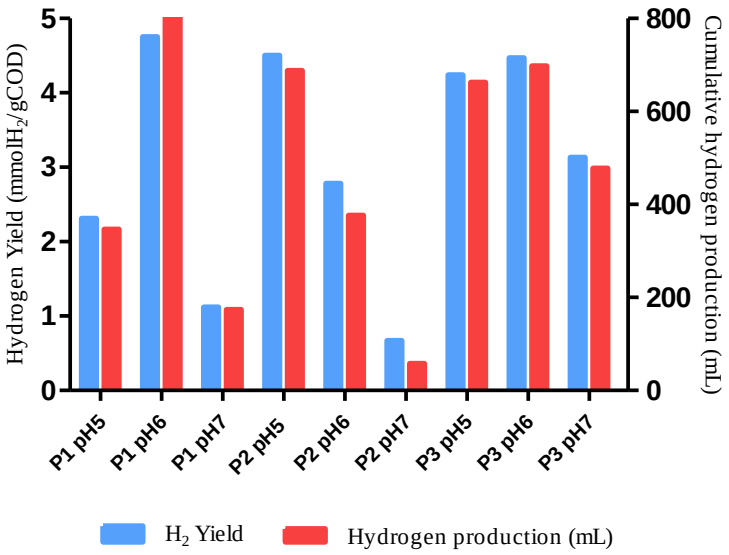

Figure 1: Hydrogen yield and cumulative hydrogen production for different initial pH and different pretreatment of inoculum.

Table 1- Kinetic parameters estimated by the modified Gompertz model. The values presented are mean of triplicate experiments; the standard deviation of the triplicates is also shown.

Bioassays	R_m(mLH_2.h^{-1})	$H_{máx}$ (mL)	λ(h)	R^2
P1 pH6	50.39 ± 5.25	821.34 ± 61.92	23.54 ± 0.88	0.9951
P2 pH5	49.39 ± 32.01	687.58 ± 60.60	57.03 ± 11.13	0.9989
P3 pH6	17.66 ± 2.03	697.68 ± 83.07	34.79 ± 0.20	0.9892

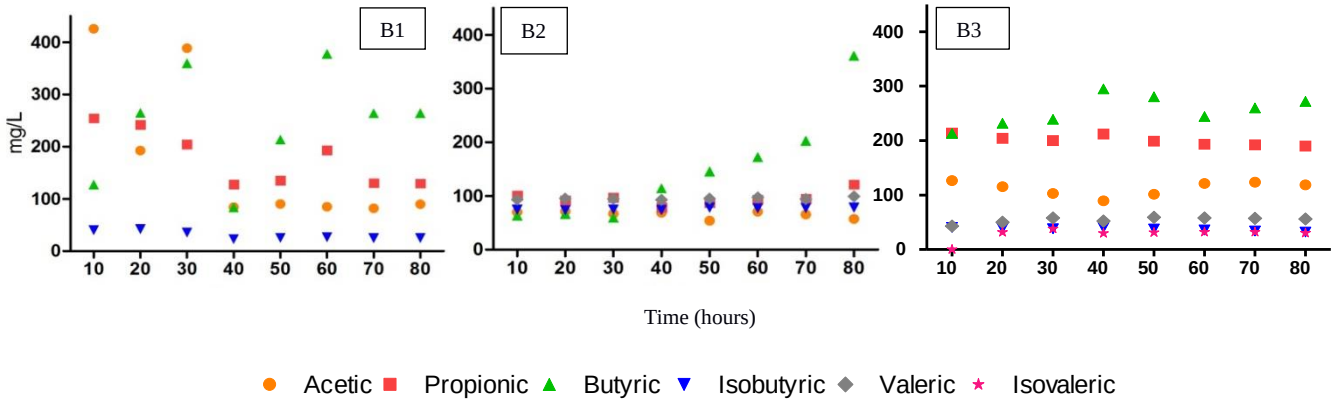

Figure 2: Production of volatile fatty acids (mg/L) in fermentation assays at different initial pH values and pretreatments of the inoculum. P1 pH6 (B1); P2 pH5 (B2) and P3 pH6 (B3).

.

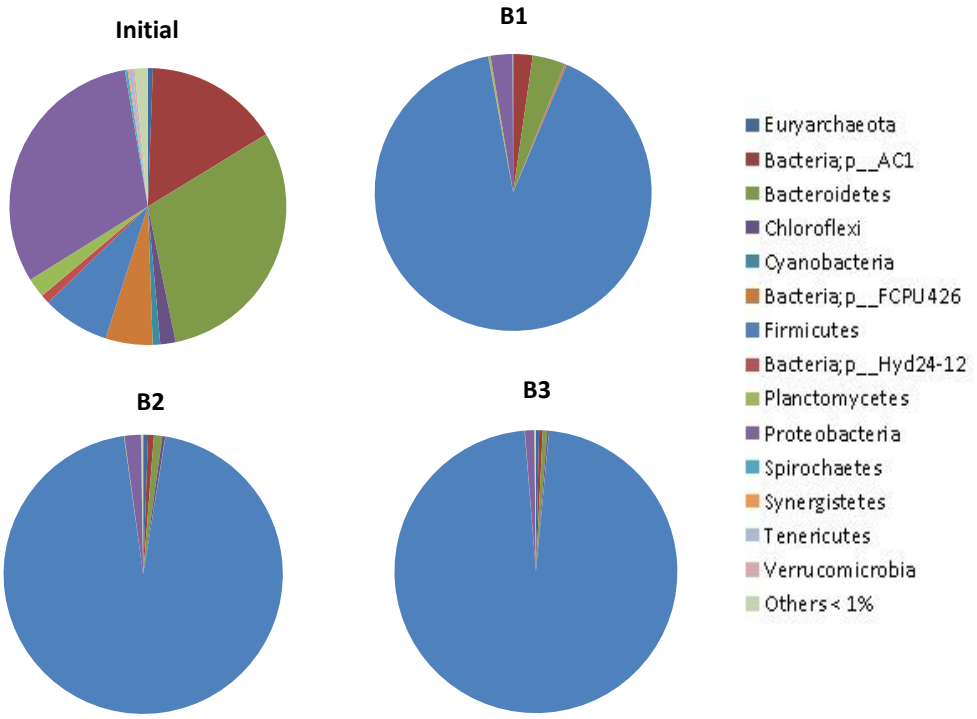

Figure 3: Taxonomic profile of the microbial communities classified at the phylum level according to the analysis of the 16S ribosomal gene of the initial inoculum and in different batch reactor tests: P1 pH6 (B1); P2 pH5 (B2) and P3 pH6 (B3).

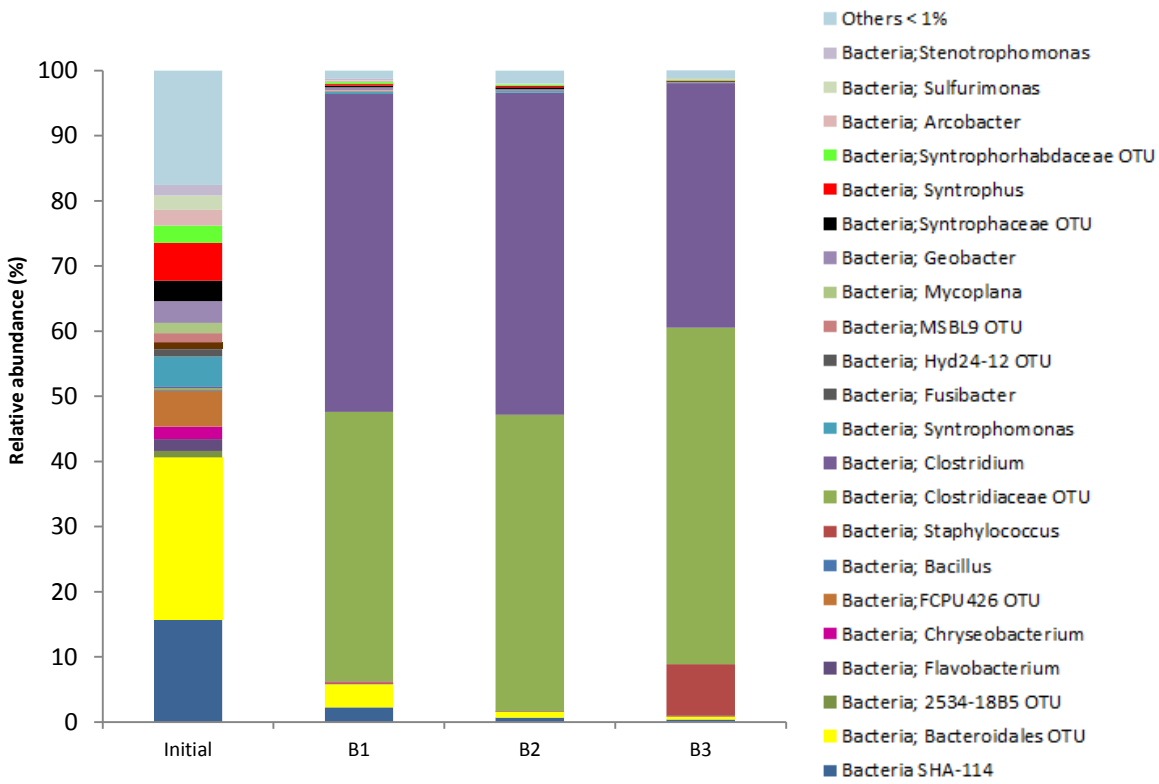

Figure 4: Relative abundance profiles of the predominant genera of the microbial communities classified according to the analysis of the 16S ribosomal gene of the initial inoculum and in different tests: B1 (P1 pH6), B2 (P2 pH5) and B3 (P3 pH6).

Table 2: OTUs detected in different batch reactor tests: B1 (P1 pH6), B2 (P2 pH5) and B3 (P3 pH6).

Phylum	**Class**	**Order**	**Family**	**Genus**	**Function in fermentation**	**Reference**
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	Acidogênese	Baek et al. (2015)
Bacteroidetes	Bacteroidia	Bacteroidales	_	_	Hidrólise / acidogênese	Yang et al. (2016)
AC1	SHA-114	_	_	_	Função desconhecida	-
Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	*Syntrophomonas*	Hidrólise / Degradação de propionato e butirato	Gan et al. (2012)
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	Hidrólise	Shah et al. (2016)
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	Formação de substância polimérica extracelular (EPS) para a formação dos grânulos	Hung et al. (2011)
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	-	Oxidação de ácidos graxos de cadeia curta	Yang et al. (2017)
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	_	Acetogênese	Si et al. (2016)
Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	*Geobacter*	Redutora de ferro	Kato et al. (2012)
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*	Acetogênese	Dhar et al. (2015)
Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	*Syntrophus*	Degradação de ácidos graxos e compostos aromáticos	McInerney et al.(2007)
FCPU426	_	_	_	_	Função desconhecida	Kuroda et al. (2016)
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	Acidogênese; Degradação de compostos orgânicos e aminoácidos	Ruiz et al. (2014)
Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Sulfurimonas*	Oxidação de enxofre em sulfato	Zhang et al. (2015)
Planctomycetes	Phycisphaerae	MSBL9	_	_	Hidrólise / acidogênese	Tsitko et al. (2014)
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Mycoplana*	Degradação de compostos aromáticos	Gao et al.(2017)
Bacteroidetes	Bacteroidia	Bacteroidales	p-2534-18B5	_	Degradação de proteínas	Slezak et al. (2017)
Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	*Chryseobacterium*	Hidrólise	Wang et al. (2016)
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	Degradação de Compostos orgânicos complexos	Gao et al.(2017
Firmicutes	Clostridia	Clostridiales	Acidaminobacteraceae	*Fusibacter*	Redutoras de tiossulfato e halotolerantes	Li et al (2008)
Hyd24-12	_	_	_	_	Função desconhecida	Shu et al. (2015)

Table 3: Microbial diversity indexes (Chao 1, Shannon and Simpson) of the initial inoculum and in different batch reactor tests: B1 (90°C/10min pH6), B2 (105°C/120 min pH5), B3 (121°C/20min pH 6).

Assays	Alpha diversity index		
	Chao 1	**Shannon (H')**	**Simpson (S)**
Initial	128	3,143	0,8983
B1	100	1,192	0,5889
B2	71	1,003	0,5485
B3	71	1,099	0,5864

Figure 5: Canonical correlation analysis (CCA) associating the most abundant bacterial genera (or respective OTUs) and chemical parameters in different tests in batch reactors: B1, B2, and B3.

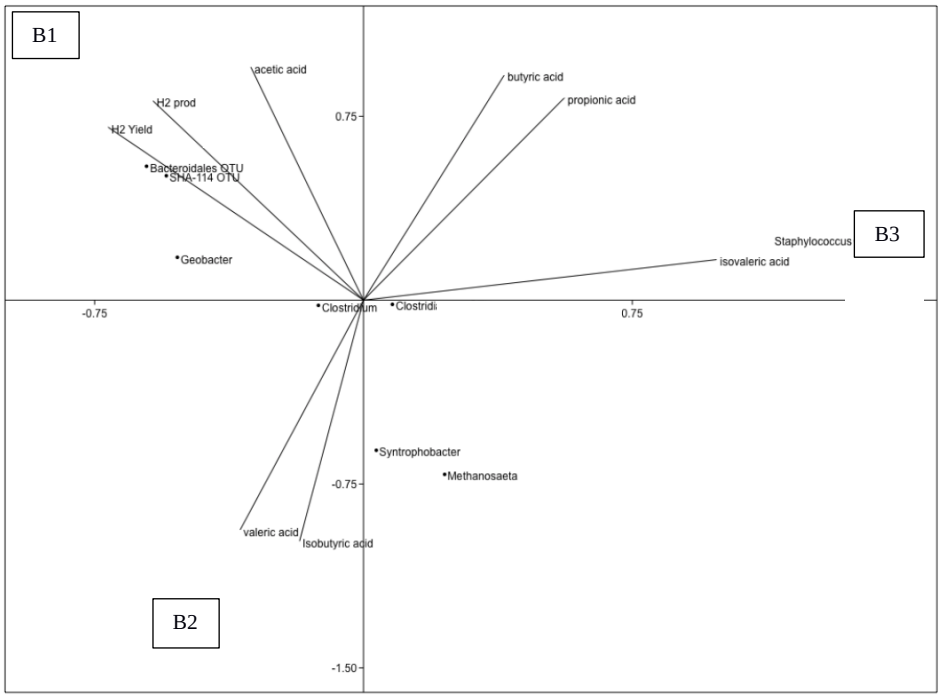

Table 4: Hydrogen production and identification of the microorganisms isolated in the different assays (B1, B2, and B3).

	Ident.	N° de acess	Microrganism	H_2 production (mL)
B1	98%	NR 113323.1	*Clostridium bifermentans*	435.07 ± 5.97
B1/B2/B3	98%	NR 102516.1	*C. saccharoperbutylacetonicum*	171.97 ± 10.84
B2/B3	100%	NR 122061.1	*C. saccharobutylicum*	160.52 ± 15.84
Co-culture	-	-	*C. saccharoperbutylacetonicum* + *C.bifermentans*	529.50 ± 8.83
	-	-	*C. saccharoperbutylacetonicum* + *C. saccharobutylicum*	34.63 ± 1.31
	-	-	*Clostridium bifermentans* + *C. saccharobutylicum*	42.75 ± 3.95
	-	-	*C. bifermentans* + *C. saccharobutylicum* + *C.saccharoperbutylacetonicum*	66.90 ± 6.41

Table 5: Percentage of OTUs of predominant fungi detected in the initial inoculum and B1 assay (P1 pH 6)

.

Phylum	Subfile	Class	Subclasses	Order	Family	Genus	Relative abundance (%)	
							Initial	Final
Ascomycota	Pezizomycotina	-	-	-	-	-	7,4	4,6
Ascomycota	Pezizomycotina	Dothideomycetes	-	-	-	-	6,9	1,9
Ascomycota	Pezizomycotina	Eurotiomycetes	Eurotiomycetidae				4,7	3,1
Ascomycota	Pezizomycotina	Eurotiomycetes	Eurotiomycetidae	Eurotiales	Trichocomaceae	*Aspergillus*	4,5	4,0
Ascomycota	Saccharomycotina	Saccharomycetes	-	Saccharomycetales		*Candida*	24,6	47,2
Basal_fungi	Mucoromycotina	-	-	-	-	-	0,4	1,5
Basal_fungi	Mucoromycotina					*Mucor*	0,3	1,2
Basidiomycota	Agaricomycotina	Agaricomycetes	-	-	-	-	4,7	5,2
Ascomycota	Saccharomycotina		Saccharomycetes	Saccharomycetales	Incertae_Sedis	*Yarrowia*	0,4	0,3

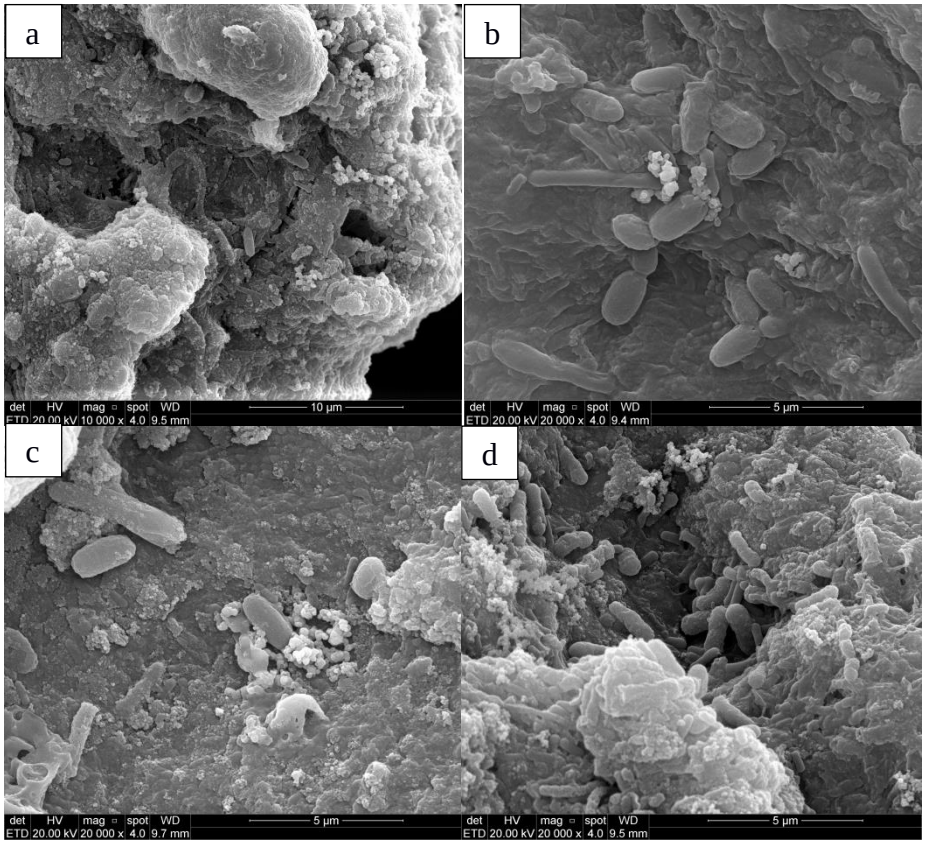

Figure 6: MEV images of the initial inoculum 10,000X (a), B1 20,000X (b), B2 20,000X (c) and B3 20,000X (d).

5. DISCUSSÃO GERAL

Este trabalho mostrou a possibilidade da utilização de vinhaça pura, sem adição de nutrientes sintéticos ou fontes de carbono adicionais, para a produção de hidrogênio e de ácidos graxos voláteis. É sabido que a utilização da vinhaça *in natura* pode dar instabilidade ao processo de digestão anaeróbia, devido à presença de alguns compostos que podem ser tóxicos. Entretanto, os esforços na utilização da vinhaça bruta são extremamente importantes, uma vez que este resíduo é altamente poluente e é gerado em altas proporções diariamente, e a utilização sem adição de outros componentes torna o processo vantajoso a ser empregado futuramente em reatores em grande escala.

Na produção de hidrogênio, os métodos de pré-tratamento do inóculo são fundamentais no processo, uma vez que promovem a eliminação de bactérias consumidoras de hidrogênio e favorecem os microrganismos capazes de produzirem esporos, como os *Clostridium*. Os três métodos de tratamento térmico avaliados neste trabalho foram eficazes na eliminação de arqueas metanogênicas e mostraram influir sobre a microbiota e consequentemente no favorecimento de rotas metabólicas importantes na produção de hidrogênio e de ácidos graxos voláteis. Assim como os distintos valores iniciais de pH (5, 6 e 7) mostraram selecionar distintas comunidades microbianas no processo fermentativo mostrando diferentes rendimentos dos produtos avaliados.

O pré-tratamento térmico do inóculo a 90°C (10 min) é bastante descrito na literatura para a produção de hidrogênio. E, neste estudo, mostrou-se mais efetivo que os demais pré-tratamentos avaliados (105°C, 120 min e 121°C, 20 min), com uma predominância de ácido butírico e consequentemente uma maior produção de hidrogênio, quando avaliado em pH 6. Os ensaios em pH 7 favoreceram a produção de ácidos graxos voláteis, evidenciando que o pH inicial da fermentação desempenha um papel fundamental no processo e o pH ótimo para hidrogênio é diferente do pH ótimo para a produção de ácidos orgânicos.

A utilização de consórcios microbianos na produção de hidrogênio é um processo bastante complexo, devido a diversidade microbiana existente no consórcio (bactérias e fungos) atuando em sinergismo na degradação do substrato e produção de gases e compostos orgânicos. O aumento da temperatura dos três pré-tratamentos do inóculo avaliados, diminuiu a diversidade microbiana e aumentou a predominância do filo Firmicutes, um dos principais envolvidos na produção de hidrogênio. No entanto, isto não incrementou a produção de hidrogênio, visto que no ensaio de melhor desempenho (90°C, 10min – pH 6) foram favorecidos tanto os microorganismos anaeróbios estritos (*Clostridium*), produtores de

hidrogênio, como os microrganismos anaeróbios facultativos (*Bacillus*, *Syntrophomonas*, *Geobacter*), que são importantes na manutenção da anaerobiose e hidrólise do substrato, mostrando que a interação entre os microrganismos é fundamental para o sucesso da fermentação. Tal fato foi confirmado nos ensaios em culturas puras com microrganismos anaeróbios estritos e anaeróbios facultativos, onde a produção de hidrogênio não apresentou o mesmo desempenho do que o consórcio microbiano.

Além da comunidade bacteriana presente no consórcio, adicionalmente foi avaliado, por sequenciamento de alto desempenho, a presença de fungos que também são importantes no processo de fermentação, pois são capazes de sintetizar enzimas que digerem polissacarídeos, atuando na hidrólise do substrato, facilitando a absorção pelas bactérias e favorecendo a produção de hidrogênio e ácidos graxos voláteis. Alguns trabalhos sugerem a bioaumentação de inóculos com fungos especializados na degradação de substratos lignocelulósicos, capazes de converter polissacarídeos em açúcares simples que podem ser posteriormente fermentados, melhorando a eficiência e rendimento do processo fermentativo.

Os estudos apresentados neste trabalho mostram a influência dos pré-tratamentos térmicos e do pH inicial da fermentação sobre a microbiota na bioconversão da vinhaça para a produção de hidrogênio e de ácidos graxos voláteis. A luz da necessidade de buscar formas de aproveitamento da vinhaça *in natura*, este trabalho mostra possibilidades para seu aproveitamento para a obtenção de diferentes produtos, hidrogênio ou ácidos, dependendo das condições de fermentação, dando um destino sustentável para este resíduo da produção de etanol gerado em grandes quantidades diariamente, reduzindo seu poder poluente e podendo ser utilizado posteriormente como fertilizante, diminuindo o impacto ambiental.

6. CONCLUSÕES

Este estudo avaliou a melhor forma de pré-tratamento do inóculo para a produção de hidrogênio a partir da degradação da vinhaça, visando a eliminação das bactérias consumidoras de hidrogênio. Adicionalmente, este estudo isolou e identificou os microrganismos presentes nos consórcios bem como os coprodutos de interesse biotecnológico. Diante dos resultados obtidos, foi possível concluir que:

O aumento na concentração de vinhaça afetou positivamente a produção de hidrogênio, e a maior produção de hidrogênio foi alcançada com a vinhaça sem diluição e sem qualquer outro suplemento adicional.

Tanto o pré-tratamento do inóculo quanto o pH inicial desempenharam um papel importante na seleção de diferentes comunidades com distintas capacidades para produzir H_2 e AGV, bem como na taxa de remoção de DQO e no consumo do substrato dos ensaios fermentativos.

De todos os experimentos realizados, o ensaio com o pré-tratamento T1 (90 °C por 10 min), foi o melhor para produzir hidrogênio em pH inicial 6. Enquanto o pré-tratamento T3 (121°C por 20 min), foi o mais eficiente para produzir AGV em pH 7.

Nos ensaios de melhor produção de hidrogênio (T1, T2 e T3), a rota metabólica butírica foi predominante. No entanto, em pH 6 a concentração destes ácidos foi maior do que no ensaio em pH 5.

Com o aumento da temperatura dos pré-tratamentos dos inóculos, houve aumento da predominância do filo Firmicutes e consequentemente a diminuição da diversidade microbiana nestes ensaios.

No ensaio de melhor produção de hidrogênio (T1), através das análises por sequenciamento de alto desempenho, encontrou-se grande abundância do gênero *Clostridium* e também a presença *Bacillus*, *Syntrophomonas*, *Geobacter*, *Arcobacter*, *Stenotrophomonas* e, Bacteroidales. Além dos fungos (Agaricomycetes, Pezizomycotina, e *Aspergillus*) descritos como importantes no processo hidrolítico da vinhaça. As condições deste ensaio (T1)

favorecem tanto os microrganismos estritos (*Clostridium*), quanto os anaeróbios facultativos (bactérias e fungos), que são extremamente importantes na manutenção da anaerobiose e hidrólise do substrato, indicando que não só estrutura microbiana é importante, mas também a abundância e a interação entre os diferentes microrganismos é o que impulsiona o processo e aumenta a eficiência na produção de hidrogênio.

Foi possível isolar uma pequena proporção da diversidade microbiana dos inóculos pré-tratados por meio de isolamento direto em placas em condições subaeróbicas e também pelo método de *Roll tube* em condições anaeróbias, obtendo-se os gêneros *Enterobacter, Bacillus* e *Clostridium.* Observamos que estes microrganismos isoladamente não possuem o mesmo desempenho na produção de H_2 que quando associados com outras espécies presentes no consórcio microbiano, o que indica mais uma vez que a interação com outros microrganismos é fundamental para o sucesso da fermentação. No entanto, esses isolados podem ser importantes para construir consórcios microbianos artificiais para incrementar a produção de H_2 e também de AGV.

Os resultados deste trabalho mostraram a influência dos pré-tratamentos térmicos do inóculo e do pH inicial na bioconversão da vinhaça de cana-de-açúcar *in natura*, na produção de hidrogênio e ácidos graxos voláteis, destacando a importância ambiental da produção de energia a partir deste resíduo da produção de biocombustível gerada diariamente em grandes quantidades.

7. PERSPECTIVAS

A partir dos resultados experimentais deste estudo, sugere-se:

- Avaliar a bioaumentação do consórcio microbiano de lodo granular com as diferentes espécies de microrganismos isolados neste trabalho, utilizando vinhaça como substrato para a produção de H_2 e AGV;
- Construir consócios microbianos sintéticos a partir dos microrganismos isolados utilizando vinhaça como substrato para a produção de H_2 e AGV;
- Comparar os resultados obtidos neste estudo com ensaios nas mesmas condições utilizando glicerol residual e outros resíduos agroindustriais;
- Ampliar os conhecimentos de metabolômica, para melhor entendimento

das alterações dos metabólitos produzidos pelos consórcios microbianos com melhor produção de H_2.

8. BIBLIOGRAFIA COMPLEMENTAR

Albanez, R.; Lovato, G.; Zaiat, M.; Ratusznei, S.M.; Rodrigues, J.A.D. (2016). Optimization, metabolic pathways modeling and scale-up estimative of an AnSBBR applied to biohydrogen production by co-digestion of vinasse and molasses. **International Journal of Hydrogen Energy**, 41: 20473-20484.

Amaral, P.F.F.; Ferreira, T.F.; Fontes, G.C.; Coelho M.A.Z. (2009). Glycerol valorization: New biotechnological routes. **Food and Bioproducts Processing**, 78:179-186.

Apha (2005). **Standart methods for the examination of water and wastewater**. 20th edition. Washington: American Public Health Associatin.

Atasoy, M.; Owusu-Agyeman, I.; Plaza, E.; Cetecioglu, Z. (2018). Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. Bioresource Technology, 268: 773–786.

Bengtsson, S.; Hallquist, J.; Werker, A., Welander, T. (2008). Acidogenic fermentation of industrial wastewaters: effects of chemostat retention time and pH on volatile fatty acids production. **Biochem. Eng. J.**, 40:492–499.

Buitrón, G.; Carvajal, C. (2010). Biohydrogen production from Tequila vinasses in an anaerobic sequencing batch reactor: Effect of initial substrate concentration, temperature and hydraulic retention time**. Bioresource Technology**, 23:9071-9077.

Castelló, E.; Braga, L.; Fuentes, L.; Etchebehere, C. (2018). Possible causes for the instability in the H_2 production from cheese whey in a CSTR. International Journal of Hydrogen Energy, 43:2654-2665.

Chang, J.J.; Wu, J.H.; Wen, F.S.; Hung, K.Y.; Chen, Y.T.; Hsiao, C.L.; Lin, C.Y.; Huang, C.C. (2008). Molecular monitoring of microbes in a continuous hydrogen-producing system with different hydraulic retention time. **International Journal of Hydrogen Energy**, 33: 1579-1585.

Chen, S.D.; Sheu, D.S.; Chen, W.M.; Lo, Y.C.; Huang, T.I.; Lin, C.Y.; Chang, J.S. (2007). Dark hydrogen fermentation from hydrolyzed starch treated with recombinant amylase originating from *Caldimonas taiwanensis* On1. **Biotechnology Progress**, 23: 1312-1320.

Cheong, D.Y.; Hansen, C.L. (2006). Bacterial stress enrichment enchances anaerobic hydrogen production in cattle manure sludge. **Applied Microbiology Biotechnology**, 72: 635-643.

Chong, M. L.;Rahman. N.A.A.; Yee, F.L.; Aziz, S.A.; Rahim, R.A.;Shirai, Y.; Hassan, M.A. (2009). Effects of pH, glucose and iron sulfate concentration on the yield of biohydrogen by *Clostridium butyricum* EB6. **International Journal of Hydrogen Energy,** 34:8859-8865.

Coral, J.; Karp, S.G.; Vandenberghe, L.P.S.; Parada, J.L.; Pandey, A.; Soccol, C.R. (2008). Batch fermentation model of propionic acid production by *Propionibacterium acidipropionici* in different carbon sources. **Appl Biochem Biotechnol.**, 151:333–341.

Dabrock B.; Bahl H.; Gottschalk, G. (1992). Parameters affecting solvent production by *Clostridium pasteurianum*. **Applied Environmental Microbiology**, 58:1233–1239.

Dahiya, S.; Sarkar, O.; Swamy, Y.V.; Venkata Mohan, S. (2015). Acidogenic fermentation of food waste for volatile fatty acid production with co-generation of biohydrogen. **Bioresource Technology**, 182: 103-113.

Das, D.; Khanna, N.; Dasgupta, C. (2008). Biohydrogen production: fundamentals and technology advances. **International Journal of Hydrogen Energy,** 33:7349-7357.

Das, D.; Veziroglu, T. N. (2008). Advances in biological hydrogen production processes. **International Journal of Hydrogen Energy**, 33, 6046-6057.

Davila-Vazquez, G.; Cota-Navarro, C.B.; Rosales-Colunga, L.M.; León-Rodríguez, A.; Razo-Flores, E (2009). Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate. **International Journal of Hydrogen Energy**, 34:4296 – 4304.

Drapcho, C.N.; Nhuan, N.P.; Walker, T. (2008). **Biofuels engineering process technology**. The United States of America: TheMcGraw-Hill Companies, Inc. 371p.

Edgar RC. (2010). Search and clustering orders of magnitude faster than BLAST. **Bioinformatics**, 26:2460-2461.

Ercolini, D. (2013). High-Throughput sequencing and metagenomics: Moving forward in the culture-independent analysis of food microbial ecology. **Appl Environ Microbiol.** 79: 3148-3155.

Ewan B.C.R.; Allen R.W.K. (2005). A figure of merit assessment of the routes to hydrogen. **International Journal of Hydrogen Energy**, 30:809–819.

Fang, H.H.P.; Liu, H.; Zhang, T. (2002). Characterization of a hydrogen-producing granular sludge. **Biotechnology and Bioengineering**, 78: 44-52.

Fang, H.H.P.; Zhang, T.; Li, C. (2006). Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge. **Journal of Biotechnology**, 126: 357-364.

Fernandes, B.S.; Peixoto, G.; Albrecht, F.R.; Aguila, N.K.S.; Zaiat, M. (2010). Potential to produce biohydrogen from various wastewaters. **Energy for Sustainable Development**, 14:143–148.

Fonseca, B.C (2016). Produção de hidrogênio por fermentação por um novo isolado de *Clostridium beijerinckii*. **Dissertação de Mestrado**. Departamento de Química, Universidade de São Paulo, Ribeirão Preto, SP.

Fonseca, J.D.; Camargo, M.; Commenge, J.M.; Falk, L.; Gil, I.D. (2019) Trends in design of distributed energy systemsusing hydrogen as energy vector: A systematicliterature review. **International Journal of Hydrogen Energy**, 44: 9486-9504.

Fraga, G.P.; Abreu, C.A.; Benetti, J.M. (1994). **Poluição do solo e aquífero subterrâneo pela vinhaça infiltrada sob tanques de armazenamento**. São Paulo: CETESB. 52p.

Fu, Z.; Holtzapple, M.T. (2010). Anaerobic mixed-culture fermentation of aqueous ammonia-treated sugarcane bagasse in consolidated bioprocessing. **Biotechnol. Bioeng**, 106: 216–227.

Fuentes, L.; Braga, L.; Castelló, E.; Etchebehere, C. (2018). Work scheme to isolate the different micro-organisms found in hydrogen-producing reactors: a study of effectiveness by pyrosequencing analysis. **Journal of Applied Microbiology**, 125:96—110.

Granato, E.F. (2003). Geração de energia através da biodigestão anaeróbica da vinhaça. **Dissertação de mestrado**. Faculdade de Engenharia, Universidade Estadual Paulista. Bauru, São Paulo, Brasil.

Gullo, M.; Verzelloni, E.; Canonico, M. (2014). Aerobic submerged fermentation by acetic acid bacteria for vinegar production: Process and biotechnological aspects. **Process Biochemistry**, 49: 1571–1579.

Guo, A.; Guo, L.; Cao, C.; Yin, J.; Lu, Y.; Zhang, X. (2012) Hydrogen production from glycerol by supercritical water gasification in a continuous flow tubular reactor. **International Journal of Hydrogen Energy**, 37: 5559-5568.

Hallenbeck, P.C. (2012). **Fundamentals of dark hydrogen fermentations: multiple pathways and enzymes**. In: Azbar, N.; Levin, D.B. (2012). State of the art and progress in production of biohydrogen (Eds). Bentham Science Publishers. 94-111.

Han, W.; Wang, Z.; Chen, H.; Yao, X.; Li, Y. (2011). Simultaneous biohydrogen and bioethanol production from anaerobic fermentation with immobilized sludge. J**ournal of Biomedicine and Biotechnology**, 2011: 343791, 5 pag.

Huang, Y.L.; Wu, Z.; Zhang, L.; Cheung, M.C.; Yang, ST. (2002). Production of carboxylic acids from hydrolyzed corn meal by immobilized cell fermentation in a fibrous-bed bioreactor. **Bioresource Technology**, 82:51-59.

Huo, Z.; Fang, Y.; Yao, G.; Zeng, X.; Ren, D.; Jin, F. (2015). Improved two-step hydrothermal process for acetic acid production from carbohydrate biomass. **Journal of Energy Chemistry**, 24:207–212.

Infantes, D.; González del Campo, A.; Villasenõr, J.; Fernández, F.J. (2011). Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation. **International Journal of Hydrogen Energy**, 36:15595-15601.

Jiang, L.; Wang, J.; Liang, S.; Wang, X.; Cen, P.; Xu, Z. (2009). Butyric acid fermentation in a fibrous bed bioreactor with immobilized *Clostridium tyrobutyricum* from cane molasses. **Bioresource Technology**, 100: 3403–3409.

Kapdan, I.; Kargi, F. (2006). Bio-hydrogen production from waste materials. **Enzyme and Microbial Technology,** 38:569-582.

Kim, D.K.; Rathnasingh, C.; Song, H.; Lee, H.J.; Seung, D.; Chang, Y.K. (2013). Metabolic engineering of a novel *Klebsiella oxytoca* strain for enhanced 2,3-butanediol production. **Journal of Bioscience and Bioengineering**, 116:186-192.

Kim, D.H.; Han, S.K.; Kim.; S.H.; Shin, H.S. (2006). Effect of gas sparging on continuous fermentative hydrogen production. **International Journal of Hydrogen Energy**, 31:2158-2169.

Kraemer, J.T.; Bagley D.M. (2007). Improving the yield from fermentative hydrogen production. **Biotechnology Letters,** 29:685-695.

Kośmider, A.; Drożdżyńska, A.; Blaszka, K.; Leja, K.; Czaczyk, K. (2010). Propionic acid production by *Propionibacterium freudenreichii* ssp. *shermanii* using crude glycerol and whey lactose industrial wastes. **Polish J. of Environ. Stud.**, 19: 1249-1253.

Kotay, S; Das, D. (2008). Biohydrogen as a renewable energy resource - Prospects and potentials. **International Journal of Hydrogen Energy**, 33:258-263.

Lay, J.; Lee, J. Y.J.; Noike, T. (1999). Feasibility of biological hydrogen production from organic fraction of municipal solid waste. **Water Research**, 33: 2579-2586.

Lay, C.H.; Wu, J.H.; Hsiao, C.L.; Chang, J.J.; Chen, C.C.; Lin, C.H. (2010). Biohydrogen production from soluble condensed molasses fermentation using anaerobic fermentation. **International Journal of Hydrogen Energy,** 35:13445-13451.

Lazaro, C. Z.; Perna, V.; Etchebehere, C.; Varesche, M. B. A. (2014). Sugarcane vinasse as substrate for fermentative hydrogen production: The effects of temperature and substrate concentration. **International Journal of Hydrogen Energy**, 39**:** 6407-6418**.**

Lazaro, C.Z.; Varesche, M.B.A.; Silva, E.L. (2015). Effect of inoculum concentration, pH, light intensity and lighting regime on hydrogen production by phototrophic microbial consortium. **Renewable Energy**, 75: 1-7.

Lee, W.S.; Chua, A.S.M.; Yeoh, H.K.; Ngoh, G.C. (2014). A review of the production and applications of waste-derived volatile fatty acids. **Chemical Engineering Journal**, 235:83–99.

Levin, D.B.; Azbar, N. (2012). **Introduction: Biohydrogen in perspective. State of the art and progress in production of biohydrogen** (Eds). Bentham Science Publishers. 3-7.

Levin, D.B; Islam, R.; Cicek, N.; Sparling, R. (2006). Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. **Process Biochemestry,** 31:1496-1503.

Levin, D.B.; Pitt, L.; Love, M. (2004). Biohydrogen production: prospects and limitations to practical application. **International Journal of Hydrogen Energy**, 29:173-185.

Li, X.; Cai, G.; Wu, D.; Zhang, M.; Lin, C.; Lu, J. (2018). Microbial community dynamics of Dan'er barley grain during the industrial malting process. **Food Microbiology**, 76:110-116.

Lim, S., Kim, B.J.; Jeong, C.; Choi, J.; Ahn, Y.H.; Chang, H.N. (2008). Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor. **Bioresour. Technol**, 99: 7866–7874.

Liu, D. (2008). Bio-hydrogen production by dark fermentation from organic wastes and residues. **PhD Thesis.** Department of Environmental Engineering Technical, University of Denmark, Denmark.

Liu, X.G.; Yang, S.T. (2006). Butyric acid and hydrogen production by *Clostridium tyrobutyricum* ATCC 25755 and mutants. **Enzyme Microb.Technol,** 38:521–528.

Liu, Y.; Liang, T.; Yuan, X.; Lv, Y. (2019). The performance of COD removal and hydrogen production in a singlestage system from starch using the consortium PB-Z under simulatednatural conditions. Energy, 173:951-958.

Lu, Y.; Slater, F.R.; Mohd-Zaki, Z.; Pratt, S.; Batstone, D.J. (2011). Impact of operating history on mixed culture fermentation microbial ecology and product mixture. **Water Sci. Technol**, 64:760–765.

Maintinguer, S.I. (2009). Obtenção e caracterização filogenética de consórcio bacteriano utilizado em reator anaeróbio em batelada aplicado à produção de hidrogênio. **Tese de doutoramento**. Departamento de hidráulica e Saneamento, Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos.

Maintinguer, S.I.; Fernandes, B.S.; Duart, I.C.C.; Saavedra, N.K.; Adorno, M.A.T.; Varesche, M.B. (2008). Fermentative hydrogen production by microbial consortium. **International Journal of Hydrogen Energy,** 33:4309-4317.

Maintinguer, S.I.; Sakamoto, I.K.; T. Adorno, M.A.; A. Varesche, M.B. (2015). Bacterial diversity from environmental sample applied to bio-hydrogen production. **International Journal of Hydrogen Energy**, 40: 3180-3190.

Matsunaga, T.; Hatano, T.; Yamada, A.; Matsumoto, M. (2000). Microaerobic hydrogen production by photosynthetic bacteria in a double-phase photobiorreator. **Biotechnology Bioengineering**, 68:647-651.

Meyer, J.; Gagnon, J. (1991). Primary structure of hydrogenase I from *Clostridium pasteurianum*. **Biochemistry**, 30:9697–9704.

Mocali, S.; Landi, S.; Curto, G.; Dallavalle, E.; Infantino, A.; Colzi, C.; d'Errico, G.; Roversi, P.F.; D'Avino, L.; Lazzeri, L. (2015). Resilience of soil microbial and nematode communities after biofumigant treatment with defatted seed meals. **Industrial Crops and Products**, 75:79–90.

Moraes, B.S.; Junqueira, T.L.; Pavanello, L.G.; Cavalett, O.; Mantelatto, P.E.; Bonomi, A.; Zaiat, M. (2014). Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? **Applied Energy**, 113:825-835.

Morimoto, K.; Kimura, T.; Sakka, K.; Ohmiya, K. (2005). Overexpression of a hydrogenase gene in Clostridium paraputrificum to enhance hydrogen gas production. **FEMS Microbiology Letters**, 246: 229-234.

Motte, J.C.; Trably, Eric.; Hamelin, J.; Escudié, R.; Bonnafous, A.; Steyer, J.P.; Bernet, N.; Delgenès, J.P.; Dumas, C. (2014). Total solid content drives hydrogen production through microbial selection during thermophilic fermentation. **Bioresource Technology**, 166:610–615.

Muyzer, G.; De Waal, E.C.; Uitterlinder, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electroforesis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. **Applied Environmental Microbiology**, 59: 695– 700.

Nielsen A.T.; Liu, W.T.; Filipe, C.; Grady, L.; Molin, S.; Stahl, D.A. (1999). Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. **Applied Environmental Microbiology**, 65:1251-1258.

Nogueira, C.E.C.; Souza, S.N.M.; Micuanski, V.C.; Azeedo, R.L. (2015). Exploring possibilities of energy insertion from vinasse biogas in the energy matrix of Paraná State, Brazil. **Renewable and Sustainable Energy Reviews**, 48:300–305.

Noori, M.; Saady, C. (2013). Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unresolved challenge. **International Journal of Hydrogen Energy**, 38:13172–13191.

Novais, C.M; Alves, M.P. (2004). PCR em Tempo Real. **Biotecnologia Ciência & Desenvolvimento**, 33:10-13.

Onuki, M.; Satoh, H.; Mino, T.; Matsuo, T. (2000). Application of molecular methods to microbial community analysis of activated sludge. **Water Science and Technology**, 42:17-22.

Parsaee, M.; Kiani, M.K.D.; Karimi, K. (2019). A review of biogas production from sugarcane vinasse. **Biomass and Bioenergy**, 122:117-125.

Perna, V.; Castelló, E.; Wenzel, J.; Zampol, C.; Fontes Lima, D.M.; Borzacconi, L.; Varesche, M.B.; Zaiat, M.; Etchebehere, C. (2013). Hydrogen production in an upflow anaerobic packed bed reactor used to treat cheese whey. **International Journal of Hydrogen**, 38:54-62.

Playne, M.J. (1985). **Propionic and butyric acids**. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology. New York: Pergamon Press. pp. 731–759.

Raganati, F.; Olivieri, G.; Gotz, P.; Marzocchella, A.; Salatino, P. (2015). Butanol production from hexoses and pentoses by fermentation of *Clostridium acetobutylicum*. **Anaerobe**, 34: 146-155.

Ratti, R.P.; Delforno, T.P.; Sakamoto, I.K.; Varesche, M.B.A. (2015). Thermophilic hydrogen production from sugarcane bagasse pretreated by steam explosion and alkaline delignification. **International Journal of Hydrogen Energy**, 40: 6296-6306.

Riesenfeld, C. S.; Schloss, P. D.; Handelsman, J. (2004). Metagenomics: genomic analysis of microbial communities. **Annu Rev Genet**, 38: 525-552.

Rodriguez, J.; Kleerebezem, R.; Lema, J.M.; van Loosdrecht, M.C.M. (2006). Modeling product formation in anaerobic mixed culture fermentations. **Biotechnol. Bioeng**, 93:592–606.

Rosa, P.R.F.; Santos, S.C.; Sakamoto, I.K.; Varesche, M.B.A.; Silva, E.L. (2014). Hydrogen production from cheese whey with ethanol-type fermentation: Effect of hydraulic retention time on the microbial community composition. **Bioresource Technology**, 161: 10-19.

Rossi, D.M.; Costa, J.B.; Souza, E.A.; Peralba, M.C.R.; Samios, D.; Ayub, M.A.Z. (2011). Comparison of different pretreatment methods for hydrogen production using environmental microbial

consortia on residual glycerol from biodiesel. **International Journal of Hydrogen Energy**, 36:4814-4819.

Sá, L.R.V.; Cammarota, M.C.; Ferreira-Leitão, V.S.; (2014). Produção de hidrogênio via fermentação anaeróbia – aspectos gerais e possibilidade de utilização de resíduos agroindustriais brasileiros. **Química Nova**, 37: 857-867.

Sá, L.R.V.; Oliveira, T.C.; Santos, T.F.; Matos, A.; Cammarota, M.C.; Oliveira, E.M.M.; Ferreira-Leitão, V.S. (2011). Hydrogenase activity monitoring in the fermentative hydrogen production using heat pretreated sludge: A useful approach to evaluate bacterial communities performance. **International Journal of Hydrogen Energy**, 36: 7543-7549.

Salomon, K. R.; Lora, E. E. S. (2009). Estimate of the electric energy generating potential for different sources of biogas in Brazil. **Biomass and bioenergy**, 33: 1101–1107.

Santangelo, J.D.; Durre, P.; Woods, D.R. (1995). Characterization and expression of the hydrogenase-encoding gene from Clostridium acetobutylicum P262. **Microbiology**, 141:171–180.

Sarah, M.; Carver, M.C.; Lepistö, R. N.; Yu,Z.; Tuovinen, O.H. (2012). Hydrogen and volatile fatty acid production during fermentation of cellulosic substrates by a thermophilic consortium at 50 and 60°C. **Bioresource Technology**, 104: 424–431.

Sans, C.; Mata-Alvarez, J.; Cecchi, F.; Pavan, P.; Bassetti, A. (1995). Volatile fatty acids production by mesophilic fermentation of mechanically-sorted urban organic wastes in a plug-flow reactor, **Bioresour. Technol**, 51:89–96.

Seibel, L.F.B.; Lemos, M.; Lifschitz, S. (2000) Bancos de dados de genoma. In: **Procs. Of the Brazilian Databases Symposium Tutorials**. P. 514-553.

Sena Oliveira, M.C.; Reginato, L.C.; Roese, A.D.; Anthonisen, D.G.; Patrocínio, E.; Parma, M.M.; Scagliusi, S.M.M.; Timóteo, W.H.; Jardim, S.N. (2007). Fundamentos teórico-prático e protocolos de extração e de amplificação de DNA por meio de reação em cadeia polimerase. **Disponível (online).** http://www2.cppse.embrapa.br/080servicos/070publicacaogratuita/e-book/LivroProtMolecular.pdf. (14 de julho de 2015).

Sinha, P.; Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. **International Journal of Hydrogen Energy**, 36: 7460-7478.

Sivagurunathan, P.; Sen, B.; Lin, C.Y. (2014). Batch fermentative hydrogen production by enriched mixed culture: Combination strategy and their microbial composition. **Journal of Bioscience and Bioengineering,** 117:222-228.

Springer, A.; Goissis G. (1988). Design of pond systems for treatment of ethanol plant effluents**. Biological Wastes,** 23:143-152.

Suhaimi, S.N.; Phang, L.Y.; Maeda, T.; Abd-Aziz, S.; Wakisaka, M.; Shirai, Y.; Hassan, M.A.(2012).Bioconversion of glycerol for bioethanol production using isolated *Escherichia coli* SS1. **Brazilian Journal of Microbiology**, 43:506-516.

Sydney, E.B.; Larroche, C.; Novak, A.C.; Nouaille, R.; Sarma, S.J.; Brar, S.K.; Letti, L.A.J.; Soccol, V. T.; Soccol, C.R. (2014). Economic process to produce biohydrogen and volatile fatty acids by a mixed culture using vinasse from sugarcane ethanol industry as nutrient source. **Bioresource Technology**, 159:380-386.

Tamagnini, P.; Axelsson, R.; Lindberg, P.; Oxelfelt, F.; Wunschiers, R.; Lindblad, P. (2002). Hydrogenases and hydrogen metabolism in cyanobacteria. **Microbiol Mol Biol Rev**, 66:1–20.

Tan, H.W.; Abdul Aziz, A.R.; Aroua, M.K. (2013). Glycerol production and its applications as a raw material: A review. **Renewable and Sustainable Energy Reviews**, 27:118–127.

Tansksale, A.; Beltramini, J.N.; Lu, G.M.A. (2010). Review of Catalytic Hydrogen Production Processes from Biomass. **Renewable and Sustainable Energy Reviews**, 14:166-182.

Uyar, B.; Eroglu, I.; Yücel, M.; Gündüz, U. (2009). Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. **International Journal of Hydrogen Energy**, 34: 4517-4523.

Vandák, D.; Telgarsky, M.; Sturdík, E. (1995). Influence of growth factor supplements on butyric acid production from sucrose by *Clostridium butyricum*. **Folia Microbiol**, 40:669–672.

Verdinelli, V.; Juan, A.; German, E. (2019). Ruthenium decorated single walled carbon nanotube for molecular hydrogen storage: A first-principle study. **International Journal of Hydrogen Energy**, 44:8376-8383.

Vignais, P.M.; Colbeau, A. (2004). Molecular Biology of Microbial Hydrogenases. **Current Issues in Molecular Biology**, 6: 159-88.

Wang, A.; Gao, L.; Ren, N.; Xu, J.; Liu, C.; Lee, D.J. (2010). Enrichment strategy to select functional consortium from mixed cultures: Consortium from rumen liquor for simultaneous cellulose degradation and hydrogen production. **International Journal of Hydrogen Energy**, 35:13413–13418.

Wang, J., Yin, Y. (2017). Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. **International Journal of Hydrogen Energy**, 42:4804-4823.

Wang, J.; Wan W. (2008). Effect of Fe^2 concentration on fermentative hydrogen production by mixed cultures. **International Journal of Hydrogen Energy,** 33:1215-1220.

Wang, J., Wan, W. (2009). Factors influencing fermentative hydrogen production: A review. **International Journal of Hydrogen Energy**, 34:799-811.

Xiao, B.; Han, Y.; Liu, J.(2010). Evaluation of biohydrogen production from glucose and protein at neutral initial pH. **International Journal of Hydrogen Energy,** 35:6152-6160.

Yildiz, I. (2018). 1.12 Fossil Fuels. Comprehensive Energy Systems, 1: 521-567.

Yossan, S.; O-Thong, S.; Prasertsan, P. (2012). Effect of initial pH, nutrients and temperature on hydrogen production from palm oil mill effluent using thermotolerant consortia and corresponding microbial communities. **International Journal of Hydrogen Energy**, 37: 13806-13814.

Zhang, K.; Ren, N.Q.; Wang, A.J. (2015). Fermentative hydrogen production from corn stover hydrolyzate by two typical seed sludges: Effect of temperature. **International Journal of Hydrogen Energy,** 40:3838-3848.

Zhao, X.; Xing, D.; Zhang, L.; Ren, N. (2010). Characterization and overexpression of a [FeFe]-hydrogenase gene of a novel hydrogen-producing bacterium Ethanoligenens harbinense. **International Journal of Hydrogen Energy**, 35: 9598-9602.