UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

FACULTAD DE INGENIERÍA CIVIL



EFFECT OF BIOLOGICAL PRETREATMENT OF LIGNOCELLULOSIC RESIDUES ON H_2 YIELD AND VFAs PRODUCTION THROUGH DARK FERMENTATION

Por

ROBERTO CARLOS CAMPOS FLORES

Como requisito parcial para obtener el grado de

DOCTORADO EN INGENIERÍA CON ORIENTACIÓN EN INGENIERÍA AMBIENTAL

NOVIEMBRE DE 2026

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École doctorale GAIA – Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau
Portée par

Unité de recherche : Laboratoire de Biotechnologie de l'Environnement (INRAE)

EFFECT OF BIOLOGICAL PRETREATMENT OF LIGNOCELLULOSIC RESIDUES ON H_2 YIELD AND VFAs PRODUCTION THROUGH DARK FERMENTATION

Présentée par Roberto Carlos CAMPOS FLORES
Le 05 novembre 2026

Sous la direction de Nicolas BERNET
et Arquímedes CRUZ LÓPEZ

Devant le jury composé de

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Dr. José Manuel Mendoza Rangel
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San Nicolás de los Garza, Nuevo León, noviembre de 2026

RESUMEN

En las últimas décadas se ha observado un deterioro medioambiental constante debido, en gran medida, al uso desmedido de combustibles fósiles que, en 2026, siguen siendo la fuente de energía predominante a nivel mundial. Debido a lo anterior, la investigación en torno a nuevas fuentes de energía limpia se ha intensificado.

El hidrógeno (H_2) como vector energético ha atraído especial interés en las últimas décadas debido a su gran capacidad energética y a su potencial de fungir como fuente de energía sin la generación de gases de efecto invernadero durante su combustión. El H_2 producido a partir de procesos biológicos es de especial interés, ya que, actualmente, el H_2 producido se obtiene mayormente de fuentes fósiles. Entre los métodos biológicos de obtención de H_2 más interesantes, se encuentra la fermentación oscura, presentando múltiples ventajas frente a otros métodos como sus mayores rendimientos de H_2, simplicidad, versatilidad en cuanto al uso de sustratos y a su relativamente fácil escalamiento. Sin embargo, esta metodología presenta ciertos desafíos que afrontar, especialmente cuando se trabaja con sustratos complejos como biomasa lignocelulósica, impactando directamente en la eficiencia del proceso observando bajos rendimientos, inestabilidad del sistema y una pobre conversión de la biomasa hacia H_2 y ácidos grasos volátiles (AGVs), específicamente ácido acético y ácido butírico.

Debido a lo anterior, múltiples métodos de pretratamiento han sido evaluados con el objetivo de hacer más eficiente el proceso cuando se usa biomasa lignocelulósica como sustrato, contando con pretratamientos químicos, físicos y biológicos. Sin embargo, muchos de estos pretratamientos presentan desventajas como su alto costo, sobreconsumo energético y, en el caso de algunos pretratamientos químicos, la generación de compuestos nocivos para la fermentación oscura. Es por esto que, en el presente trabajo, se evaluó la producción de H_2 y AGVs a través del proceso de fermentación oscura utilizando residuos lignocelulósicos (cáscaras de frutas, grano gastado de cerveza y paja de trigo) como sustrato, usando hongos de pudrición blanca (*Trametes versicolor* y

Pleurotus ostreatus) como método de pretratamiento biológico, buscando mejorar la eficiencia del proceso de fermentación oscura a través de un pretratamiento económico, amigable con el medio ambiente, sin sobre consumo de energía ni generación de compuestos tóxicos.

El principal objetivo de la investigación fue promover la fermentación de azúcares reductores presentes en la biomasa lignocelulósica mediante el pretratamiento con hongos de pudrición blanca para mejorar la producción de H_2 en la fermentación oscura. Evaluando diferentes condiciones de pretratamiento en la fermentación en estado sólido como temperatura, cepa de hongo y tiempo de fermentación mediante la implementación de un diseño factorial mixto 3x2x2, evaluando tres niveles de tiempo (15, 25 y 35 días), dos niveles de temperatura (28 y 32°C) y dos diferentes especies de hongos (*T. versicolor* y *P. ostreatus*), teniendo como respuesta la concentración de azúcares reductores (AR) (buscando tener la mayor concentración posible de este parámetro) y la concentración de lignina ce Klason (buscando observar una mayor deslignificación después del pretratamiento). Acoplando así los bioprocesos de fermentación en estado sólido (FES) y fermentación oscura (FO).

Para lograr dichos objetivos, se montaron diversos experimentos empezando por un par de BHP test (biohydrogen production potential, por sus siglas en inglés), en donde primeramente se evaluó una mezcla de sustratos con una proporción de 50% p/p grano gastado de cerveza (GGC), 20% p/p cáscara de banana (CB), 20% p/p cáscara de melón (CM) y 10% p/p cáscara de piña (CP), utilizando condiciones específicas en la fermentación oscura como el uso de lodo granular anaerobio pretratado térmicamente (100°C, 30 min) como inóculo, un ratio de inoculación (S/I) de 2.7, pH inicial de 5.50 ± 0.05. El segundo BHP test montado, denominado en las siguientes secciones como modified BHP test (ensayo de BHP modificado), consistió en el uso de una nueva mezcla lignocelulósica conformada por 20% p/p GGC, 20% p/p CB, 20% p/p CM, 20% p/p CP y la adición de 20% p/p paja de trigo (PT), operado a diferentes condiciones de FO, utilizando ahora como inóculo lodo aerobio pretratado térmicamente (100°C, 30 min) a una relación S/I 20, y una

concentración de sólidos volátiles (SV) iniciales de <10 $g \cdot L^{-1}$. Posterior a los ensayos de BHP se montaron dos reactores de 3 L con un volumen de trabajo de 2 y 1.5 L, operados en modalidad batch y batch secuencial, respectivamente, contando en cada experimento con el grupo control (sustrato crudo) y el grupo de estudio, con sustrato pretratado bajo las condiciones establecidas después del análisis en el diseño factorial mixto aplicado en la etapa de pretratamiento.

En la etapa de pretratamiento, utilizando la primera mezcla de residuos lignocelulósicos establecida, después de analizar los resultados del diseño factorial mixto 3x2x3, se observó un mejor comportamiento en términos de concentración de azúcares reductores y de degradación de lignina en el pretratamiento llevado a cabo con *T. versicolor* a 28 °C durante 25 días, observando un ligero incremento en la concentración de AR del día 15 al 25 de 9.48 a 33.75 $g \cdot kg^{-1}$ y una reducción de lignina de Klason desde el día 0 al 25 de 15%. Por otro lado, se observó que *P. ostreatus* consumía más AR que *T. versicolor* en todos los tiempos de muestreo bajo todas las condiciones evaluadas, indicando que dicho hongo sigue una ruta de degradación no selectiva, consumiendo todos los componentes de la lignocelulosa por igual, haciéndolo ineficiente para el objetivo de la investigación.

Posterior a la determinación de las condiciones de pretratamiento idóneas, se empleó el sustrato pretratado bajo dichas condiciones en el primer BHP test, utilizando botellas serológicas de 124 mL con un volumen de trabajo de 80 mL bajo las condiciones de operación previamente establecidas, montando dos grupos de reactores: Control (C-H) y utilizando el sustrato previamente hidrolizado por *T. versicolor* en la etapa de pretratamiento (TV-H). La reacción se detuvo hasta observar la fase estacionaria en la producción de H_2, después de 72 h, en donde se observó que el grupo C-H presentó una mayor producción acumulada de biogás (1775 ± 70.60 $mL \cdot L^{-1}$), sin embargo, la producción de H_2 en este grupo fue menor a la observada en el TV-H, alcanzando una producción acumulada de tan solo 166 ± 1 $mL \cdot L^{-1}$ después de las 72 horas. Lo anterior fue debido a que, pese a haber obtenido una mayor producción de biogás, la proporción de H_2 en biogás fue menor en el C-H en todos los tiempos de muestreo en contraste con el TV-H, donde se

observó una producción acumulada de H_2 de 341 ± 1 $mL \cdot L^{-1}$. Los anteriores resultados indican que, pese a haber obtenido una menor concentración de AR iniciales en el TV-H (1.97 ± $g \cdot L^{-1}$ vs. 12.96 ± 1.36 $g \cdot L^{-1}$ en el C-H), la acumulación de H_2 no se vio afectada, sino la acumulación de biogás en general, observando una clara influencia entre la concentración inicial de AR y la producción de biogás. No obstante, la composición de biogás fue únicamente H_2 y CO_2 en ambos grupos de reactores, mostrando que el pretratamiento térmico aplicado al inóculo resultó efectivo inhibiendo microorganismos metanogénicos.

Adicional a la mejora en producción de H_2, se observó de igual manera una mejora importante en el rendimiento de este, pasando de 7 $mLH_2 \cdot gVS^{-1}$ en el C-H a 19 $mLH_2 \cdot gVS^{-1}$, representando una mejora de 2.71 veces con respecto al grupo control. De igual manera, la acumulación de AGVs resultó ser mayor en el TV-H, alcanzando valores de 0.133 $g \cdot gVS^{-1}$ comparado con una producción acumulada de tan solo 0.034 $g \cdot gVS^{-1}$ en el C-H, aunque siendo en ambos casos el ácido acético y ácido butírico los subproductos predominantes.

Los anteriores resultados respecto al mejorado rendimiento de H_2 y acumulación de AGVs al final del proceso en el grupo TV-H se vieron también reflejados en la composición de comunidades microbianas analizadas a las 72 h. Al final de la fermentación oscura, el sistema pretratado con *T. versicolor* (TV-H) fue dominado exclusivamente por bacterias productoras de H_2 (BPH), con una abundancia relativa del 79% del OTU relacionado con el género *Klebsiella*, 13% para el OTU relacionado con *Clostridium* y 3% para *Escherichia*. Estos resultados indicaron que el pretratamiento fúngico realizado a través de la FES por *T. versicolor* durante 25 días a 28 °C no solo mejoran la eficiencia de la FO en términos de producción de H_2 y acumulación de AGVs, sino que también alteran positivamente la composición de las comunidades microbianas presentes en los reactores, y, por consiguiente, influencian las rutas metabólicas dentro del sistema, promoviendo la acumulación de ácido acético y ácido butírico.

Sin embargo, pese a haber observado mejoras importantes en todos los indicadores de eficiencia en la FO durante el ensayo de BHP, el rendimiento de H_2

obtenido, aun cuando se utilizó el sustrato pretratado, seguía siendo muy bajo (19 $mLH_2 \cdot gVS^{-1}$). Por ende, se llevó a cabo el segundo ensayo de BHP, denominado "modified BHP test", en donde se observaron mejoras sustanciales en términos de rendimiento de H_2, producción acumulada de AGVS y comunidades microbianas mediante el ajuste de los parámetros de operación previamente descritos en el presente texto.

Siguiendo dichas modificaciones, y montando nuevamente dos grupos de estudio en el modified BHP test con sustrato crudo (RAW MIX) y pretratado por *T. versicolor* bajo las condiciones dictaminadas en la etapa de pretratamiento (SSF25d MIX), el rendimiento de H_2 logró mejorar hasta 81 ± 9 $mLH_2 \cdot gVS^{-1}$ en el grupo SSF25d MIX y hasta 42 ± 7 $mLH_2 \cdot gVS^{-1}$ en el grupo control (RAW MIX), una vez más demostrando el positivo impacto del pretratamiento fúngico del sustrato en la eficiencia del proceso de FO.

Adicionalmente, en este caso, el pH del sistema fue monitoreado al inicio y al final de la fermentación oscura. El grupo SSF25d MIX mostró una capacidad buffer mejorada en contraste con el grupo RAW MIX, teniendo como resultado una menor acidificación en el medio al final del proceso de FO que, en este caso, duró 42 h. El pH inicial de ambos grupos de estudio fue de 5.50 ± 0.08, mientras que después de 42 h de reacción, el grupo RAW MIX mostró un pH de 4.40 ± 0.08, mientras que en el grupo SSF25d MIX, el pH inicial observado fue de 5.04 ± 0.14. Adicionalmente, en este modified BHP test, se observó la misma tendencia de mejora en cuanto a la producción acumulada de AGVs al final del proceso en el SSF25d MIX, alcanzando una acumulación total de 0.429 $g \cdot gVS^{-1}$, mientras que en el grupo RAW MIX la acumulación total fue de 0.109 $g \cdot gVS^{-1}$, siendo el ácido acético y el ácido butírico los únicos subproductos detectados en ambos grupos.

En cuanto a las comunidades microbianas detectadas al final del proceso en el modified BHP test, se observó la misma tendencia de mejora en el porcentaje de abundancia relativa de BPH cuando se trabaja con el sustrato pretratado con *T. versicolor*, ya que, en el grupo SSF25d MIX se observó una predominancia del 70% del OTU relacionado con el género *Clostridium* y un 9% del OTU relacionado con el

género *Klebsiella*, siendo los dos géneros con mayor abundancia relativa en dicho sistema. Por otro lado, el RAW MIX presentó una abundancia relativa del 60% de *Clostridium*, seguida de un 19% de *Lactobacillus*, un género bacteriano generalmente asociado a rutas metabólicas no productoras de H_2.

Lo anterior nuevamente confirmó el impacto positivo del pretratamiento en la proliferación de microorganismos productores de H_2 y, adicionalmente, se observó de igual manera una mejora en los porcentajes de abundancia relativa del OTU relacionado con *Clostridium* en este segundo BHP test, logrando en este experimento una mejor eficiencia del proceso no solo en el rendimiento de H_2, sino también en la acumulación de AGVs y la diversidad en las comunidades microbianas.

Posterior a estos resultados positivos, y al haber observado una mejora importante en la eficiencia del proceso en el modified BHP test, utilizando como sustrato la segunda mezcla de residuos lignocelulósicos establecida, se procedió a realizar un escalamiento del proceso en reactores batch y, posteriormente, en batch secuenciales, replicando las mismas condiciones de operación del modified BHP test (pH inicial de 5.50 ± 0.08, SV iniciales < 10 $g \cdot L^{-1}$, 150 rpm de agitación, S/I 20 usando logo aerobio pretratado térmicamente como inóculo). Se montaron, nuevamente, dos diferentes reactores operados en modalidad batch conteniendo sustrato crudo (RBR) y sustrato pretratado por *T. versicolor* (FPBR) y dos reactores en modalidad batch secuencial (RSBR, usando sustrato crudo y FPSBR, usando sustrato pretratado). Sin embargo, pese a haber obtenido resultados muy favorables en los previos ensayos de BHP, cuando el proceso se escaló en estos reactores batch de 3 L con un volumen de trabajo de 2 L para el caso de los reactores batch y de 1.5 L para los reactores continuos, se observaron rendimientos de H_2muy bajos en comparación con los previos sistemas estudiados, obteniendo rendimientos de tan solo 20 a 34 $mLH_2 \cdot gVS^{-1}$ en los sistemas batch y batch secuenciales pretratados con *T. versicolor*, respectivamente, mientras que los reactores usando sustrato crudo operados bajo la misma modalidad obtuvieron rendimientos de H_2 de 97 a 195

$mLH_2 \cdot gVS^{-1}$, respectivamente. Evidenciando un efecto negativo del pretratamiento en el rendimiento de H_2 en sistemas más grandes con pH controlado.

Esta tendencia también se vio observada en la producción acumulada de AGVs al final del proceso batch y al final de cada ciclos de alimentación en el batch secuencial (operado durante 192 horas con tres ciclos de alimentación), observando una mayor concentración de AGVS en los sistemas alimentados con sustrato sin pretratamiento (RBR y RSBR) con concentraciones yendo desde 0.300 hasta 0.350 $g \cdot gVS^{-1}$, mientras que en los sistemas alimentados con sustrato pretratado (FPBR y FPSBR), las concentraciones totales de AGVs oscilaron entre 0.120 y 0.290 $g \cdot gVS^{-1}$.

Adicionalmente, el enriquecimiento de la abundancia relativa de BPH previamente observado en los sistemas de BHP alimentados con sustrato pretratado no se reflejó en este caso. En contraste, los reactores batch y batch secuenciales con sustrato crudo (RBR y RSBR) exhibieron una mayor abundancia relativa de BPH que los reactores batch y batch secuenciales con sustrato pretratado (FPBR y FPSBR). Particularmente, el OTU afiliado con el género *Clostridium* se vio favorecido en los reactores RBR y RSBR, mientras que su abundancia relativa fue menor en los FPBR y FPSBR. En su lugar, se observó una mayor proliferación del OTU relacionado con el género *Enterobacter* en estos sistemas. Aunque las bacterias del género *Enterobacter* tienen la capacidad de producir H_2, su eficiencia es generalmente menor que la de las bacterias del género *Clostridium*, lo cual pudiera explicar parcialmente el menor rendimiento de H_2 observado en el FPBR y FPSBR.

El fenómeno anterior puede ser atribuido a diversos factores asociados con el escalamiento del proceso desde pequeña escala (80-200 mL) hasta reactores más grandes con un volumen de trabajo de 1.5 y 2 L. Los factores potenciales que pudieron haber influido en esto, incluyen la formación de gradientes de pH y oxígeno dentro de los sistemas operados en reactores de 3 L de capacidad. Adicionalmente, en todos los casos el sustrato pretratado con *T. versicolor* presentó concentraciones

de AR considerablemente menores que las de los sustratos crudos, lo cual pudo haber afectado negativamente la eficiencia del proceso en escalas más grandes.

Una hipótesis adicional a este deterioro de eficiencia observado cuando el sistema fue operado a mayores escalas es el factor de remoción de oxígeno en dichos sistemas. En los reactores batch y batch secuenciales se realizó una purga con gas N_2 durante 1 minuto, lo cual pudo no haber sido suficiente para establecer condiciones estrictamente anaerobias al tratarse de reactores con capacidad de 3 L, en contraste con los sistemas de BHP que eran sustancialmente más pequeños, utilizando frascos de menores capacidades (124 mL y 600 mL para el BHP test y modified BHP test, respectivamente). Por lo que, oxígeno residual pudo haber quedado presente en los reactores más grandes, afectando negativamente el desarrollo de las comunidades microbianas productoras de H_2, y, por ende, la eficiencia general en el proceso de fermentación oscura.

Esta limitación pudo haber afectado particularmente a los reactores alimentados con sustrato pretratado con *T. versicolor*, ya que el pretratamiento aerobio efectuado por dicho organismo pudo haber propiciado la retención de oxígeno residual en el sustrato y el micelio generado durante el pretratamiento. Además, el incremento en la porosidad del sustrato generada durante el pretratamiento fúngico pudo haber facilitado la retención de oxígeno en la biomasa pretratada. Esta hipótesis se sustenta con el análisis de las comunidades microbianas en los sistemas alimentados con sustrato pretratado, las cuales revelan una presencia significativa del OTU asociado al género *Enterobacter* después de 48 h de operación. Dado que las especies de *Enterobacter* son anaerobias facultativas, su proliferación pudiera representar un indicador biológico de oxígeno residual dentro de los sistemas FPBR y FPSBR.

Adicional a los ensayos realizados para determinar el potencial de producción de H_2 de los sustratos pretratados con *T. versicolor*, se llevó a cabo un ensayo de BMP (biochemical methane potential, por sus siglas en inglés) con el objetivo de determinar la biodegradabilidad del sustrato crudo y pretratado bajo las condiciones establecidas. Para tal motivo, se empleó la segunda mezcla lignocelulósica, la cual

se utilizó en el modified BHP test y en los sistemas FPBR y FPSBR. Sin embargo, los resultados del ensayo de BMP demostraron que el pretratamiento no mejoró la biodegradabilidad del sustrato comparado con el sustrato crudo. No obstante, considerando los resultados observados en los dos BHP test montados, donde se observó una mejora importante en el rendimiento de H_2, producción acumulada de AGVs y composición microbiana, puede ser inferido que el pretratamiento fúngico mejora la biodisponibilidad más que la biodegradabilidad, Así, aunque la biodegradabilidad del sustrato pretratado fue solo marginalmente inferior a la del sustrato crudo (20.99% vs. 23.80%), los rendimientos de H_2 mejorados en los primeros dos experimentos, considerando la producción de H_2 por gramo de SV inicial, así como la mayor concentración total de AGVs al final de la reacción sugiere que el pretratamiento fúngico logró incrementar la accesibilidad de azúcares fermentables, permitiendo así el enriquecimiento de comunidades microbianas favorables para la producción de H_2 en los sistemas de BHP.

Como conclusiones generales, después de haber evaluado el comportamiento de la producción de H_2 y AGVs en los distintos sistemas montados, se puede argumentar que el pretratamiento fúngico propicia una mejor eficiencia en el proceso de fermentación oscura en términos de rendimiento de H_2 y acumulación de AGVs, pero con algunos matices.

En sistemas a pequeña escala, como en los ensayos de BHP, sin control de pH, se observó una mejora sustancial en términos de rendimiento de H_2, acumulación de AGVs y efecto buffer, sin embargo, en sistemas más grandes esta tendencia se vio revertida.

Múltiples factores pudieron haber afectado la eficiencia en el proceso en los reactores de 3 L operados en modalidad batch y batch secuencial, entre ellos destacan la insuficiente purga con N_2 al momento de iniciar el proceso y, por supuesto, la baja cantidad de azúcares reductores cuando se trabaja con sustrato pretratado.

Debido a lo anterior, para futuras investigaciones, es importante cuidar este parámetro, buscando un mínimo consumo de azúcares reductores en el proceso de

pretratamiento, ya sea ajustando condiciones de humedad, tiempo, temperatura, especie y cepa de hongo, así como un monitoreo constante del perfil enzimático secretado por el hongo empleado durante la FES.

Se observó una tendencia general (salvo en el primer ensayo de BHP efectuado) de, entre menor concentración de AR iniciales, menor acumulación de biogás. Lo anterior afectó en la mayoría de los casos a la acumulación final de H_2 en el sistema, pues, pese a haber observado una mayor proporción de H_2 en biogás en los sistemas alimentados con sustrato pretratado con *T. versicolor*, la evidente baja cantidad de biogás en dichos sistemas mermó la acumulación global de H_2.

Adicionalmente, se observó una eficiente supresión de microorganismos metanogénicos en todos los casos debido al empleo del pretratamiento térmico del inóculo, observándose en todos los casos únicamente H_2 y CO_2 en las muestras de biogás analizadas en los diversos tiempos de muestreo, incluso cuando los reactores fueron operados hasta 192 horas en los sistemas batch secuenciales.

SUMMARY

In recent decades, continuous environmental degradation has been observed, mainly due to the overreliance on fossil fuels, which still constitute the world's primary energy source in 2026. As a result, research into new clean energy sources has intensified.

Hydrogen (H_2) has attracted considerable attention as an energy carrier in recent decades because of its high energy density and its potential to provide clean energy without generating greenhouse gas emissions during combustion. H_2 produced through biological processes is of special interest because most H_2 production is still obtained from fossil sources. Among the most interesting biological methods for H_2 production is dark fermentation, which presents multiple advantages over other methods, such as higher H_2 yields, simplicity, versatility regarding substrate utilization, and its relatively easy scale-up. However, this methodology presents certain challenges, especially when working with complex substrates such as lignocellulosic biomass, directly impacting process efficiency by resulting in low yields, system instability, and poor biomass conversion into H_2 and volatile fatty acids (VFAs), specifically acetic acid and butyric acid.

Due to the above, multiple pretreatment methods have been evaluated with the aim of making the process more efficient when lignocellulosic biomass is used as a substrate, including physical, chemical, and biological pretreatments. However, many of these pretreatments present disadvantages such as high costs, excessive energy consumption, and, in the case of some chemical pretreatments, the generation of toxic compounds that compromise dark fermentation. Therefore, in the present work, H_2 and VFA production through the dark fermentation process was evaluated using lignocellulosic residues (fruit peels, brewer's spent grain, and wheat straw) as substrate, employing white-rot fungi (*Trametes versicolor* and *Pleurotus ostreatus*) as a biological pretreatment method, seeking to improve the efficiency of the dark fermentation process through an economical and environmentally friendly pretreatment, without excessive energy consumption or the generation of toxic compounds.

The main objective of this research was to promote the fermentation of reducing sugars present in lignocellulosic biomass through pretreatment with white-rot fungi in order to improve H_2 production during dark fermentation. Different pretreatment conditions in solid-state fermentation were evaluated, including temperature, fungal strain, and fermentation time, through the implementation of a 3x2x2 mixed factorial design. Three time levels (15, 25, and 35 days), two temperature levels (28 and 32°C), and two different fungal species (*T. versicolor* and *P. ostreatus*) were evaluated, using reducing sugars (RS) concentration (seeking to obtain the highest possible concentration of this parameter) and Klason lignin concentration (seeking to observe greater delignification after pretreatment) as response variables. Thus, coupling the bioprocesses of solid-state fermentation (SSF) and dark fermentation (DF).

To achieve these objectives, several experiments were conducted, beginning with a pair of BHP tests (biohydrogen production potential). In the first test, a substrate mixture composed of 50% w/w brewer's spent grain (BSG), 20% w/w banana peel (BP), 20% w/w melon peel (MP), and 10% w/w pineapple peel (PP) was evaluated under specific dark fermentation conditions, including the use of thermally pretreated granular anaerobic sludge (100°C, 30 min) as inoculum, a substrate-to-inoculum ratio (S/I) of 2.7, and an initial pH of 5.50 ± 0.05. The second BHP test, referred to in the following sections as the modified BHP test, consisted of the use of a new lignocellulosic mixture composed of 20% w/w BSG, 20% w/w BP, 20% w/w MP, 20% w/w PP, and the addition of 20% w/w wheat straw (WS), operated under different dark fermentation conditions. In this case, thermally pretreated aerobic sludge (100°C, 30 min) was used as inoculum at an S/I 20 and an initial volatile solids (VS) concentration of <10 $g \cdot L^{-1}$. Following the BHP tests, two 3-L reactors with working volumes of 2 and 1.5 L were operated in batch and sequencing-batch modes, respectively. Each experiment included a control group (untreated substrate) and a study group containing substrate pretreated under the conditions established after the analysis of the mixed factorial design applied during the pretreatment stage.

During the pretreatment stage, using the first lignocellulosic residue mixture established, after analyzing the results of the 3x2x2 mixed factorial design, the best performance in terms of reducing sugar concentration and lignin degradation was observed in the pretreatment carried out with *T. versicolor* at 28°C for 25 days. Under these conditions, a slight increase in RS concentration was observed from day 15 to day 25, increasing from 9.48 to 33.75 $g \cdot kg^{-1}$, while Klason lignin content decreased by 15% from day 0 to day 25. On the other hand, *P. ostreatus* was observed to consume more RS than *T. versicolor* at all sampling times under all evaluated conditions, indicating that this fungus follows a non-selective degradation pathway, consuming all lignocellulosic components equally, making it inefficient for the objective of this research.

After determining the optimal pretreatment conditions, the substrate pretreated under these conditions was used in the first BHP test, employing 124 mL serum bottles with a working volume of 80 mL under the previously established operating conditions. Two groups of reactors were set up: a control group (C-H) and a group using the substrate previously hydrolyzed by *T. versicolor* during the pretreatment stage (TV-H). The reaction was allowed to proceed until the stationary phase of H_2 production was reached after 72 h. At this point, the C-H group exhibited a higher cumulative biogas production (1775 ± 70.60 $mL \cdot L^{-1}$). However, H_2 production in this group was lower than that observed in TV-H, reaching a cumulative production of only 166 ± 1 $mL \cdot L^{-1}$ after 72 h. This was because, despite achieving higher biogas production, the H_2 proportion in the biogas was lower in the C-H group at all sampling times compared with TV-H, where a cumulative H_2 production of 341 ± 1 $mL \cdot L^{-1}$ was observed. These results indicate that, despite the lower initial RS concentration in TV-H (1.97 $g \cdot L^{-1}$ vs. 12.96 ± 1.36 $g \cdot L^{-1}$ in C-H), H_2 accumulation was not negatively affected; rather, the overall accumulation of biogas was affected, revealing a clear influence of the initial RS concentration on biogas production. Nevertheless, biogas composition consisted exclusively of H_2 and CO_2 in both reactor groups, demonstrating that the thermal pretreatment applied to the inoculum was effective in inhibiting methanogenic microorganisms.

In addition to the improvement in H_2 production, a significant increase in H_2 yield was also observed, increasing from 7 $mLH_2 \cdot gVS^{-1}$ in the C-H group to 19 $mLH_2 \cdot gVS^{-1}$ in TV-H, representing a 2.71-fold improvement compared to the control group. Likewise, VFA accumulation was higher in TV-H, reaching values of 0.133 $g \cdot gVS^{-1}$ compared to a cumulative production of only 0.034 $g \cdot gVS^{-1}$ in C-H, although acetic acid and butyric acid were the predominant by-products in both cases.

The aforementioned results regarding the improved H_2 yield and VFA accumulation at the end of the process in the TV-H group were also reflected in the microbial community composition analyzed after 72 h. At the end of dark fermentation, the system pretreated with *T. versicolor* (TV-H) was dominated exclusively by hydrogen-producing bacteria (HPB), with a relative abundance of 79% for the OTU related to the genus *Klebsiella*, 13% for the OTU related to *Clostridium*, and 3% for *Escherichia*. These results indicated that the fungal pretreatment carried out through SSF using *T. versicolor* for 25 days at 28°C not only improved dark fermentation efficiency in terms of H_2 production and VFA accumulation, but also positively altered the composition of the microbial communities present in the reactors and, consequently, influenced the metabolic pathways within the system, promoting the accumulation of acetic acid and butyric acid.

However, despite the significant improvements observed in all dark fermentation efficiency indicators during the BHP test, the H_2 yield obtained remained very low (19 $mLH_2 \cdot gVS^{-1}$), even when the pretreated substrate was used. Therefore, a second BHP assay, referred to as the "modified BHP test", was conducted, in which substantial improvements in terms of H_2 yield, cumulative VFA production, and microbial community composition were observed through the adjustment of the operating parameters previously described in this work.

Following these modifications, and once again establishing two study groups in the modified BHP test using untreated substrate (RAW MIX) and substrate pretreated by *T. versicolor* under the conditions determined during the pretreatment stage (SSF25d MIX), H_2 yield increased to 81 ± 9 $mLH_2 \cdot gVS^{-1}$ in the SSF25d MIX group and to 42 ± 7 $mLH_2 \cdot gVS^{-1}$ in the control group (RAW MIX), once again

demonstrating the positive impact of fungal pretreatment on dark fermentation process efficiency.

Additionally, in this case, the pH of the system was monitored at the beginning and at the end of dark fermentation. The SSF25d MIX group exhibited an improved buffering capacity compared to the RAW MIX group, resulting in lower acidification of the medium at the end of the dark fermentation process, which in this case lasted 42 h. The initial pH of both study groups was 5.50 ± 0.08. However, after 42 h of reaction, the RAW MIX group exhibited a pH of 4.40 ± 0.08, whereas the final pH observed in the SSF25d MIX group was 5.04 ± 0.14. Furthermore, in this modified BHP test, the same trend of improvement in cumulative VFA production at the end of the process was observed in the SSF25d MIX group, reaching a total accumulation of 0.429 $g \cdot gVS^{-1}$, whereas the RAW MIX group reached a total accumulation of 0.109 $g \cdot gVS^{-1}$. Acetic acid and butyric acid were the only by-products detected in both groups.

Regarding the microbial communities detected at the end of the process in the modified BHP test, the same trend of improvement in the relative abundance of HPB was observed when using substrate pretreated with *T. versicolor*. In the SSF25d MIX group, a predominance of 70% was observed for the OTU related to the genus *Clostridium* and 9% for the OTU related to the genus *Klebsiella*, these being the two genera with the highest relative abundance in this system. In contrast, the RAW MIX group exhibited a relative abundance of 60% for *Clostridium*, followed by 19% for *Lactobacillus*, a bacterial genus generally associated with non-hydrogen-producing metabolic pathways.

These results once again confirmed the positive impact of fungal pretreatment on the proliferation of hydrogen-producing microorganisms. Additionally, an increase in the relative abundance of the OTU related to *Clostridium* was also observed in this second BHP test. Consequently, this experiment achieved greater process efficiency not only in terms of H_2 yield, but also in VFA accumulation and microbial community composition.

Following these positive results, and after observing a significant improvement in process efficiency during the modified BHP test using the second lignocellulosic residue mixture established, the process was scaled up in batch reactors and subsequently in sequencing-batch reactors, replicating the same operating conditions employed in the modified BHP test (initial pH of 5.50 ± 0.08, initial VS concentration <10 $g \cdot L^{-1}$, agitation at 150 rpm, and an S/I 20 using thermally pretreated aerobic sludge as inoculum). Once again, two different batch reactors were established, containing untreated substrate (RBR) and substrate pretreated with *T. versicolor* (FPBR), as well as two sequencing-batch reactors (*RSBR*, using untreated substrate, and *FPSBR*, using pretreated substrate). However, despite the highly favorable results obtained in the previous BHP assays, when the process was scaled up to these 3-L reactors with working volumes of 2 L for the batch reactors and 1.5 L for the sequencing-batch reactors, very low H_2 yields were observed compared with the previously studied systems. H_2 yields of only 20 and 34 $mLH_2 \cdot gVS^{-1}$ were obtained in the batch and sequencing-batch reactors using substrate pretreated with *T. versicolor*, respectively, whereas the reactors operated under the same conditions using untreated substrate achieved H_2 yields of 97 and 195 $mLH_2 \cdot gVS^{-1}$, respectively. These results evidenced a negative effect of the pretreatment on H_2 yield in larger-scale systems operated under controlled pH conditions.

This trend was also observed in the cumulative VFA production at the end of the batch process and at the end of each feeding cycle in the sequencing-batch reactor, which was operated for 192 h over three feeding cycles. Higher VFA concentrations were observed in the systems fed with untreated substrate (RBR and RSBR), reaching values ranging from 0.300 to 0.350 $g \cdot gVS^{-1}$, whereas the systems fed with pretreated substrate (FPBR and FPSBR) exhibited total VFA concentrations ranging from 0.120 to 0.290 $g \cdot gVS^{-1}$.

Additionally, the enrichment of the relative abundance of HPB previously observed in the BHP systems fed with pretreated substrate was not reflected in this case. In contrast, the batch and sequencing-batch reactors fed with untreated

substrate (RBR and RSBR) exhibited a higher relative abundance of HPB than the batch and sequencing-batch reactors fed with pretreated substrate (FPBR and FPSBR). In particular, the OTU affiliated with the genus *Clostridium* was favored in the RBR and RSBR reactors, whereas its relative abundance was lower in the FPBR and FPSBR reactors. Instead, a greater proliferation of the OTU related to the genus *Enterobacter* was observed in these systems. Although bacteria belonging to the genus *Enterobacter* are capable of producing H_2 , their efficiency is generally lower than that of bacteria from the genus *Clostridium*, which could partially explain the lower H_2 yields observed in the FPBR and FPSBR reactors.

The aforementioned phenomenon may be attributed to several factors associated with scaling up the process from small-scale systems (80-200 mL) to larger reactors with working volumes of 1.5 and 2 L. Potential factors that may have contributed to this behavior include the formation of pH and oxygen gradients within the systems operated in 3-L reactors. Additionally, in all cases, the substrate pretreated with *T. versicolor* exhibited considerably lower RS concentrations than the untreated substrates, which may have negatively affected process efficiency at larger scales.

An additional hypothesis for the decrease in efficiency observed when the system was operated at larger scales is related to oxygen removal within these systems. The batch and sequencing-batch reactors were purged with N_2 gas for 1 minute, however, this may not have been sufficient to establish strictly anaerobic conditions in reactors with a capacity of 3 L. In contrast, the BHP systems were substantially smaller, employing serum bottles with much lower capacities (124 and 600 mL for the BHP test and the modified BHP test, respectively). Consequently, residual oxygen may have remained in the larger reactors, potentially affecting the development of hydrogen-producing microbial communities and, therefore, the overall efficiency of the dark fermentation process.

This limitation may have particularly affected the reactors fed with substrate pretreated with *T. versicolor*, since the aerobic pretreatment carried out by this organism may have promoted the retention of residual oxygen in the substrate and

in the mycelium generated during the pretreatment. Furthermore, the increase in substrate porosity generated during the fungal pretreatment may have facilitated oxygen retention in the pretreated biomass. This hypothesis is supported by the analysis of the microbial communities in the systems fed with pretreated substrate, which revealed a significant presence of the OTU associated with the genus *Enterobacter* after 48 h of operation. Given that *Enterobacter* species are facultative anaerobes, their proliferation may represent a biological indicator of residual oxygen within the FPBR and FPSBR systems.

In addition to the assays carried out to determine the H_2 production potential of the substrates pretreated with *T. versicolor*, a BMP (biochemical methane potential) test was conducted with the objective of determining the biodegradability of the untreated and pretreated substrate under the established conditions. For this purpose, the second lignocellulosic mixture was used, which was employed in the modified BHP test and in the FPBR and FPSBR systems. However, the results of the BMP assay demonstrated that the pretreatment did not improve the biodegradability of the substrate compared with the untreated substrate. Nevertheless, considering the results observed in the two BHP tests conducted, where a significant improvement in H_2 yield, cumulative VFA production, and microbial composition was observed, it may be inferred that fungal pretreatment improves bioavailability rather than biodegradability. Thus, although the biodegradability of the pretreated substrate was only marginally lower than that of the untreated substrate (20.99% vs. 23.80%), the improved H_2 yields in the first two experiments, considering H_2 production per gram of initial VS, as well as the higher total VFA concentration at the end of the reaction, suggests that the fungal pretreatment succeeded in increasing the accessibility of fermentable sugars, thereby allowing the enrichment of microbial communities favorable for H_2 production in the BHP systems.

As a general conclusion, after evaluating the behavior of H_2 and VFA production in the different systems established, it can be argued that fungal

pretreatment promotes better efficiency in the dark fermentation process in terms of H_2 yield and VFA accumulation, but with some nuances.

In small-scale systems, such as the BHP assays, without pH control, a substantial improvement was observed in terms of H_2 yield, VFA accumulation, and buffering effect; however, in larger systems this trend was reversed.

Multiple factors may have affected process efficiency in the 3-L reactors operated in batch and sequencing-batch modes, among which insufficient N_2 purging at the beginning of the process and the low amount of reducing sugars when working with pretreated substrate stand out.

Due to the above, for future research, it is important to take care of this parameter, seeking a minimum consumption of reducing sugars during the pretreatment process, either by adjusting moisture, time, temperature, fungal species, and fungal strain conditions, as well as through constant monitoring of the enzymatic profile secreted by the fungus employed during SSF.

A general trend was observed (except in the first BHP assay conducted) that the lower the initial RS concentration, the lower the biogas accumulation. This affected, in most cases, the final H_2 accumulation in the system, since, despite having observed a higher proportion of H_2 in the biogas in the systems fed with substrate pretreated with *T. versicolor*, the evident low amount of biogas in these systems reduced the overall H_2 accumulation.

Additionally, an efficient suppression of methanogenic microorganisms was observed in all cases due to the use of thermal pretreatment of the inoculum, with only H_2 and CO_2 being observed in all biogas samples analyzed at the different sampling times, even when the reactors were operated for up to 192 h in the sequencing-batch systems.

RÉSUMÉ

Au cours des dernières décennies, on a observé une dégradation environnementale continue, principalement en raison de la dépendance excessive aux combustibles fossiles, qui restent encore la principale source d'énergie mondiale en 2026. Cette situation a intensifié la recherche de nouvelles sources d'énergie propres.

L'hydrogène a suscité un intérêt considérable en tant que vecteur énergétique ces dernières années, en raison de sa haute densité énergétique et de son potentiel à fournir une énergie propre sans générer d'émissions de gaz à effet de serre lors de la combustion. L'hydrogène produit par des procédés biologiques est d'un intérêt particulier, car, à ce jour, la plupart de la production d'hydrogène provient encore de sources fossiles. Parmi les méthodes biologiques les plus intéressantes pour la production d'hydrogène figure la fermentation sombre, qui présente plusieurs avantages par rapport à d'autres méthodes, tels que des rendements plus élevés, une simplicité d'utilisation, une grande polyvalence vis-à-vis de la gamme de substrats utilisables et une mise à l'échelle relativement aisée. Cependant, cette méthodologie pose certains défis, notamment lorsque l'on travaille avec des substrats complexes comme la biomasse lignocellulosique, ce qui impacte directement l'efficacité du procédé en entraînant de faibles rendements, une instabilité du système et une conversion médiocre de la biomasse en H_2 et en acides gras volatils (AGV), notamment l'acide acétique et l'acide butyrique.

En raison de ces constats, de nombreuses méthodes de prétraitement ont été évaluées afin de rendre le procédé plus efficace lorsque la biomasse lignocellulosique est utilisée comme substrat, incluant des prétraitements physiques, chimiques et biologiques. Cependant, nombre de ces prétraitements présentent des inconvénients tels que des coûts élevés, une consommation énergétique excessive et, dans le cas de certains prétraitements chimiques, la génération de composés toxiques compromettant la fermentation. Ainsi, dans le présent travail, la production d'hydrogène et d'AGV par le procédé de fermentation sombre a été évaluée en utilisant des résidus lignocellulosiques (écorces de fruits, drêches de brasserie et

paille de blé) comme substrat, en employant des champignons lignivores blancs (*Trametes versicolor* et *Pleurotus ostreatus*) comme méthode de prétraitement biologique, dans le but d'améliorer l'efficacité du procédé de fermentation grâce à un prétraitement économique et respectueux de l'environnement, sans consommation énergétique excessive ni génération de composés toxiques.

L'objectif principal de cette recherche était de favoriser la fermentation des sucres réducteurs présents dans la biomasse lignocellulosique grâce à un prétraitement par des champignons lignivores blancs afin d'améliorer la production d'hydrogène lors de la fermentation sombre. Diverses conditions de prétraitement en fermentation solide (SSF) ont été évaluées, incluant la température, la souche fongique et la durée de fermentation, à travers la mise en œuvre d'un plan factoriel mixte 3 x 2 x 2. Trois niveaux de durée (15, 25 et 35 jours), deux niveaux de température (28 °C et 32 °C) et deux espèces fongiques différentes (*T. versicolor* et *P. ostreatus*) ont été étudiés, en utilisant la concentration en sucres réducteurs (RS) (dans le but d'obtenir la concentration la plus élevée possible) et la concentration en lignine de Klason (dans le but d'observer une plus grande delignification après le prétraitement) comme variables réponses. Ainsi, les bioprocédés de fermentation solide (SSF) et de fermentation sombre (DF) ont été couplés.

Pour atteindre ces objectifs, plusieurs expériences ont été menées, à commencer par une paire de BHP tests. Dans le premier test, un mélange de substrat composé de 50 % w/w de drêches de brasserie (BSG), 20 % w/w d'écorce de banane (BP), 20 % w/w d'écorce de melon (MP) et 10 % w/w d'écorce d'ananas (PP) a été évalué sous des conditions spécifiques de fermentation sombre, incluant l'utilisation de boues anaérobies granulaire prétraitées thermiquement (100 °C, 30 min) comme inoculum, un rapport substrat-/-inoculum (S/I) de 2,7 et un pH initial de 5,50 ± 0,05. Le second BHP test, désigné dans les sections suivantes comme le « BHP test modifié », a consisté en l'utilisation d'un nouveau mélange lignocellulosique composé de 20 % w/w de BSG, 20 % w/w de BP, 20 % w/w de MP, 20 % w/w de PP, avec l'ajout de 20 % w/w de paille de blé (WS), exploité sous d'autres conditions de fermentation sombre. Dans ce cas, un boues aérobie

prétraitée thermiquement (100 °C, 30 min) a été utilisée comme inoculum à un rapport S/I de 20 et une concentration initiale de matières volatiles (VS) < 10 $g \cdot L^{-1}$. Après les BHP tests, deux réacteurs de 3 L avec des volumes utiles de 2 L et 1,5 L ont été exploités respectivement en mode batch et en mode SBR. Chaque expérience comportait un groupe témoin (substrat non traité) et un groupe d'étude contenant le substrat prétraité selon les conditions établies après l'analyse du plan factoriel mixte appliqué lors de l'étape de prétraitement.

Lors de l'étape de prétraitement, en utilisant le premier mélange de résidus lignocellulosiques établi, l'analyse des résultats du plan factoriel 3 x 2 x 2 a montré que la meilleure performance en termes de concentration en sucres réducteurs et de dégradation de la lignine était obtenue avec le prétraitement réalisé par *T. versicolor* à 28 °C pendant 25 jours. Sous ces conditions, on a observé une légère augmentation de la concentration en RS entre le jour 15 et le jour 25, passant de 9,48 à 33,75 $g \cdot kg^{-1}$, tandis que la teneur en lignine de Klason diminuait de 15 % entre le jour 0 et le jour 25. En revanche, *P. ostreatus* a été observé à consommer davantage de RS que *T. versicolor* à tous les temps d'échantillonnage et sous toutes les conditions évaluées, indiquant que ce champignon suit une voie de dégradation non sélective, consommant tous les composants lignocellulosiques de façon égale, ce qui le rend inefficace pour l'objectif de cette recherche.

Après avoir déterminé les conditions optimales de prétraitement, le substrat prétraité selon ces conditions a été employé dans le premier BHP test, à l'aide de flacons de sérum de 124 mL avec un volume de travail de 80 mL suivant les conditions opérationnelles préalablement établies. Deux groupes de réacteurs ont été mis en place : un groupe témoin (C-H) et un groupe utilisant le substrat auparavant hydrolysé par *T. versicolor* pendant le prétraitement (TV-H). La réaction a été laissée à se dérouler jusqu'à atteindre la phase stationnaire de production d'hydrogène après 72 h. À ce stade, le groupe C-H a présenté une production cumulative de biogaz plus élevée (1775 ± 70,60 $mL \cdot L^{-1}$). Cependant, la production d'hydrogène dans ce groupe était inférieure à celle observée dans le groupe TV-H, n'atteignant que 166 ± 1 $mL \cdot L^{-1}$ après 72 h. En effet, malgré une production de

biogaz plus importante, la proportion d'hydrogène dans le biogaz était plus basse dans le groupe C-H à tous les temps d'échantillonnage comparativement au groupe TV-H, où une production cumulative d'H_2 de 341 ± 1 mL·L^{-1} a été observée. Ces résultats indiquent que, malgré une concentration initiale en RS plus faible dans TV-H (1,97 g·L^{-1} contre 12,96 ± 1,36 g·L^{-1} dans C-H), l'accumulation d'hydrogène n'a pas été affectée ; en revanche, l'accumulation globale de biogaz l'a été, révélant une influence claire de la concentration initiale en RS sur la production de biogaz. Néanmoins, la composition du biogaz était exclusivement constituée d'H_2 et de CO_2 dans les deux groupes de réacteurs, démontrant que le prétraitement thermique appliqué à l'inoculum était efficace pour inhiber les micro-organismes méthanogènes.

En plus de l'amélioration de la production d'hydrogène, une augmentation significative du rendement d'hydrogène a été constatée, passant de 7 mL H_2·gVS^{-1} dans le groupe C-H à 19 mL H_2·gVS^{-1} dans le groupe TV-H, soit une amélioration de 2,71-fois par rapport au groupe contrôle. De même, l'accumulation d'AGVs était plus élevée dans TV-H, atteignant 0,133 g·gVS^{-1} contre une production cumulative de seulement 0,034 g·gVS^{-1} dans C-H, bien que l'acide acétique et l'acide butyrique aient été les principaux sous-produits dans les deux cas.

Les résultats précédents concernant le meilleur rendement d'hydrogène et l'accumulation d'AGVs à la fin du processus dans le groupe TV-H se sont également reflétés dans la composition de la communauté microbienne analysée après 72 h. À la fin de la fermentation sombre, le système prétraité avec *T. versicolor* (TV-H) était dominé exclusivement par des bactéries productrices d'hydrogène (HPB), avec une abondance relative de 79 % pour l'OTU liée au genre Klebsiella, 13 % pour l'OTU liée au genre Clostridium et 3 % pour Escherichia. Ces résultats indiquent que le prétraitement fongique réalisé via SSF avec *T. versicolor* pendant 25 jours à 28 °C a non seulement amélioré l'efficacité de la fermentation sombre en termes de production d'hydrogène et d'accumulation d'AGVs, mais a également modifié favorablement la composition des communautés microbiennes présentes dans les

réacteurs, influençant ainsi les voies métaboliques du système et favorisant l'accumulation d'acide acétique et d'acide butyrique.

Cependant, malgré les améliorations significatives observées sur tous les indicateurs d'efficacité de la fermentation sombre lors du BHP test, le rendement d'hydrogène obtenu restait très faible (19 mL $H_2 \cdot gVS^{-1}$), même lorsque le substrat prétraité était utilisé. Par conséquent, un second BHP test, dénommé « BHP test modifié », a été réalisé, dans lequel d'importantes améliorations en termes de rendement d'hydrogène, d'accumulation d'AGVs et de composition de la communauté microbienne ont été observées grâce à l'ajustement des paramètres opérationnels précédemment décrits dans ce travail.

Après ces modifications, et en établissant de nouveau deux groupes d'étude dans le BHP test modifié en utilisant du substrat non traité (RAW MIX) et du substrat prétraité par *T. versicolor* selon les conditions déterminées lors de l'étape de prétraitement (SSF25d MIX), le rendement d'hydrogène a augmenté à 81 ± 9 mL $H_2 \cdot gVS^{-1}$ dans le groupe SSF25d MIX et à 42 ± 7 mL $H_2 \cdot gVS^{-1}$ dans le groupe témoin (RAW MIX), démontrant à nouveau l'impact positif du prétraitement fongique sur l'efficacité du processus de fermentation sombre.

De plus, dans ce cas, le pH du système a été suivi au début et à la fin de la fermentation sombre. Le groupe SSF25d MIX a présenté une capacité tampon améliorée comparée au groupe RAW MIX, entraînant une moindre acidification du milieu à la fin du processus de fermentation, qui a duré 42 h. Le pH initial des deux groupes était de 5,50 ± 0,08. Cependant, après 42 h de réaction, le groupe RAW MIX affichait un pH de 4,40 ± 0,08, alors que le pH final observé dans le groupe SSF25d MIX était de 5,04 ± 0,14. Par ailleurs, dans ce BHP test modifié, la même tendance d'amélioration de la production d'AGVs en fin de processus a été observée dans le groupe SSF25d MIX, atteignant une accumulation totale de 0,429 g·gVS^{-1}, tandis que le groupe RAW MIX n'a totalisé que 0,109 g·gVS^{-1}. L'acide acétique et l'acide butyrique ont été les seuls sous-produits détectés dans les deux groupes.

Concernant les communautés microbiennes détectées à la fin du processus dans le BHP test modifié, la même tendance d'amélioration de l'abondance relative

des HPB a été observée lorsqu'on utilisait le substrat prétraité avec *T. versicolor*. Dans le groupe SSF25d MIX, une prédominance de 70 % a été observée pour l'OTU liée au genre *Clostridium* et de 9 % pour l'OTU liée au genre *Klebsiella*, ces deux genres étant les plus abondants dans ce système. En revanche, le groupe RAW MIX présentait une abondance relative de 60 % pour *Clostridium*, suivi de 19 % pour Lactobacillus, un genre bactérien généralement associé à des voies métaboliques non productrices d'hydrogène. Ces résultats confirment à nouveau l'impact positif du prétraitement fongique sur la prolifération de micro-organismes producteurs d'hydrogène. De plus, une augmentation de l'abondance relative de l'OTU liée à *Clostridium* a également été observée dans ce second BHP test. En conséquence, cette expérience a permis d'atteindre une plus grande efficacité du processus non seulement en termes de rendement d'hydrogène, mais aussi d'accumulation d'AGVs et de composition de la communauté microbienne.

Après ces résultats positifs, et après avoir observé une amélioration significative de l'efficacité du processus lors du BHP test modifié avec le second mélange de résidus lignocellulosiques établi, le procédé a été mis à l'échelle dans des réacteurs batch puis dans des réacteurs séquentiels discontinus (SBR), en reproduisant les mêmes conditions opérationnelles employées dans le BHP test modifié (pH initial de 5,50 ± 0,08, concentration initiale de VS < 10 $g \cdot L^{-1}$, agitation à 150 rpm et un rapport S/I de 20 utilisant un boues aérobie prétraité thermiquement comme inoculum). Une fois de plus, deux réacteurs batch distincts ont été mis en place, contenant respectivement du substrat non traité (RBR) et du substrat prétraité avec *T. versicolor* (FPBR), ainsi que deux réacteurs SBR (RSBR, utilisant du substrat non traité, et FPSBR, utilisant du substrat prétraité). Cependant, malgré les résultats très favorables obtenus dans les précédents BHP tests, lorsqu'on a mis le processus à l'échelle dans ces réacteurs de 3 L (volumes de travail 2 L pour les batch et 1,5 L pour les SBR), les rendements d'hydrogène observés étaient très faibles comparés aux systèmes étudiés précédemment. Les rendements d'hydrogène n'étaient que de 20 et 34 mL $H_2 \cdot gVS^{-1}$ dans les réacteurs batch et SBR utilisant le substrat prétraité avec *T. versicolor*, respectivement, alors que les réacteurs opérés dans les mêmes conditions avec le substrat non traité atteignaient des rendements

d'hydrogène de 97 et 195 mL $H_2 \cdot gVS^{-1}$, respectivement. Ces résultats témoignent d'un effet négatif du prétraitement sur le rendement d'hydrogène dans les systèmes de plus grande taille opérés sous contrôle du pH.

Cette tendance a également été observée concernant la production d'AGVs à la fin du batch et à la fin de chaque cycle d'alimentation dans le réacteur SBR, qui a fonctionné pendant 192 h sur trois cycles d'alimentation. Des concentrations plus élevées en AGVs ont été relevées dans les systèmes alimentés avec le substrat non traité (RBR et RSBR), atteignant des valeurs comprises entre 0,300 et 0,350 $g \cdot gVS^{-1}$, tandis que les systèmes alimentés avec le substrat prétraité (FPBR et FPSBR) présentaient des concentrations totales en AGVs comprises entre 0,120 et 0,290 $g \cdot gVS^{-1}$. De plus, l'enrichissement de l'abondance relative des HPB précédemment observé dans les systèmes BHP alimentés avec du substrat prétraité n'a pas été reflété dans ce cas. Au contraire, les réacteurs batch et SBR alimentés avec le substrat non traité (RBR et RSBR) présentaient une abondance relative de HPB plus élevée que les réacteurs batch et SBR alimentés avec le substrat prétraité (FPBR et FPSBR). En particulier, l'OTU affiliée au genre *Clostridium* était favorisée dans les réacteurs RBR et RSBR, alors que son abondance était plus faible dans les réacteurs FPBR et FPSBR. À l'inverse, une plus grande prolifération de l'OTU liée au genre *Enterobacter* a été observée dans ces systèmes. Bien que les bactéries du genre *Enterobacter* puissent produire de l'hydrogène, leur efficacité est généralement moindre que celle des bactéries du genre *Clostridium*, ce qui pourrait partiellement expliquer les rendements d'hydrogène plus bas observés dans les réacteurs FPBR et FPSBR.

Ce phénomène peut être attribué à plusieurs facteurs associés à la mise à l'échelle du procédé depuis les petits systèmes (80-200 mL) vers des réacteurs plus grands de 1,5 et 2 L. Parmi les facteurs potentiels pouvant avoir contribué à ce comportement figurent la formation de gradients de pH et d'oxygène au sein des systèmes exploités dans les réacteurs de 3 L. De plus, dans tous les cas, le substrat prétraité avec *T. versicolor* présentait des concentrations en RS nettement

inférieures à celles du substrat non traité, ce qui a pu affecter négativement l'efficacité du procédé à plus grande échelle.

Une hypothèse supplémentaire concernant la diminution d'efficacité observée lorsque le système était exploité à plus grande échelle est liée à l'élimination de l'oxygène au sein de ces systèmes. Les réacteurs batch et SBR ont été purgés avec du gaz N_2 pendant 1 minute, ce qui a pu ne pas être suffisant pour établir des conditions strictement anaérobies dans des réacteurs d'une capacité de 3 L. En revanche, les systèmes BHP étaient nettement plus petits, utilisant des flacons de sérum avec des capacités beaucoup plus faibles (124 et 600 mL pour le BHP test et le BHP test modifié, respectivement). En conséquence, il est possible qu'un résidu d'oxygène soit resté dans les réacteurs plus grands, affectant potentiellement le développement des communautés microbiennes productrices d'hydrogène et, par là même, l'efficacité globale de la fermentation sombre.

Cette limitation aurait pu affecter plus particulièrement les réacteurs alimentés avec le substrat prétraité par *T. versicolor*, car le prétraitement aérobie réalisé par cet organisme pourrait avoir favorisé la rétention d'oxygène résiduel dans le substrat et le mycélium généré lors du prétraitement. De plus, l'augmentation de la porosité du substrat générée par le prétraitement fongique aurait pu faciliter la rétention d'oxygène dans la biomasse prétraitée. Cette hypothèse est soutenue par l'analyse des communautés microbiennes dans les systèmes alimentés avec du substrat prétraité, qui a révélé une présence significative de l'OTU associée au genre Enterobacter après 48 h d'exploitation. Étant donné que les espèces du genre Enterobacter sont des anaérobies facultatives, leur prolifération peut constituer un indicateur biologique d'une présence résiduelle d'oxygène au sein des systèmes FPBR et FPSBR.

En complément des essais visant à déterminer le potentiel de production d'hydrogène des substrats prétraités par *T. versicolor*, un test BMP (potentiel biochimique de méthane) a été réalisé afin de déterminer la biodégradabilité du substrat non traité et du substrat prétraité dans les conditions établies. À cet effet, le second mélange lignocellulosique a été utilisé, celui employé dans le BHP test

modifié et dans les systèmes FPBR et FPSBR. Cependant, les résultats de l'essai BMP ont démontré que le prétraitement n'a pas amélioré la biodégradabilité du substrat par rapport au substrat non traité. Néanmoins, compte tenu des résultats observés dans les deux BHP, où une amélioration importante du rendement d'hydrogène, de la production d'AGVs et de la composition microbienne a été constatée, on peut en déduire que le prétraitement fongique améliore la disponibilité biologique plutôt que la biodégradabilité. Ainsi, bien que la biodégradabilité du substrat prétraité soit seulement légèrement inférieure à celle du substrat non traité (20,99 % vs. 23,80 %), les rendements d'hydrogène accrus observés dans les deux premiers essais, en considérant la production d'hydrogène par gramme de VS initial, ainsi que la concentration totale plus élevée en AGVs à la fin de la réaction, suggèrent que le prétraitement fongique a réussi à augmenter l'accessibilité des sucres fermentescibles, permettant ainsi d'enrichir les communautés microbiennes favorables à la production d'hydrogène dans les systèmes BHP.

En synthèse, après avoir étudié le comportement de la production d'hydrogène et d'AGVs dans les différents systèmes mis en place, on peut affirmer que le prétraitement fongique favorise une meilleure efficacité du procédé de fermentation sombre en termes de rendement d'hydrogène et d'accumulation d'AGVs, mais avec quelques nuances. Dans les systèmes de petite échelle, tels que les BHP tests (sans contrôle du pH), une amélioration substantielle a été observée en termes de rendement d'hydrogène, d'accumulation d'AGVs et d'effet tampon ; toutefois, dans les systèmes de plus grande taille, cette tendance s'est inversée. Plusieurs facteurs peuvent avoir affecté l'efficacité du procédé dans les réacteurs de 3 L en mode batch et SBR, parmi lesquels la purge insuffisante d'azote au démarrage du procédé et la faible quantité de sucres réducteurs lors de l'utilisation du substrat prétraité.

En vue de recherches futures, il est crucial de maîtriser ce paramètre, en cherchant à limiter la consommation de sucres réducteurs pendant le prétraitement, que ce soit par l'ajustement de l'humidité, du temps, de la température, des espèces

fongiques et des conditions de souche, ainsi que par le suivi constant du profil enzymatique sécrété par le champignon employé durant la SSF.

Une tendance générale a été observée (sauf lors du premier BHP test) : plus la concentration initiale en RS est basse, plus l'accumulation de biogaz est faible. Ce phénomène a affecté, dans la plupart des cas, l'accumulation finale d'hydrogène dans le système, puisque, bien que la proportion d'hydrogène dans le biogaz soit plus élevée dans les systèmes alimentés avec le substrat prétraité par *T. versicolor*, la quantité totale de biogaz était plus faible, réduisant ainsi l'accumulation globale d'H_2.

Par ailleurs, une suppression efficace des micro-organismes méthanogènes a été observée dans tous les cas grâce à l'utilisation d'un prétraitement thermique de l'inoculum, ne laissant apparaître que de l'H_2 et du CO_2 dans tous les échantillons de biogaz analysés aux différents temps d'échantillonnage, même lorsque les réacteurs ont fonctionné jusqu'à 192 h dans les systèmes SBR.

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LIST OF ACRONYMS

AD	**Anaerobic digestion**
AS	**Aerobic sludge**
BHP	**Biohydrogen production potential**
BMP	**Biomethane production potential**
BP	**Banana peel**
BRF	**Brown-rot fungi**
BSG	**Brewer's spent grain**
C-H	**Control hydrolysate**
CH_4	**Methane**
COD	**Chemical oxygen demand**
CO_2	**Carbon dioxide**
DF	**Dark fermentation**
FID	**Flame ionization detector**
FPBR	**Fungal-pretreated batch reactor**
FPSBR	**Fungal-pretreated sequencing-batch reactor**
FRP	**Fruit peels**
GAS	**Granular anaerobic sludge**
GC	**Gas chromatography**
H_2	**Hydrogen**
HPB	**Hydrogen-producing bacteria**
HPE	**Hydrogen production efficiency**
MP	**Melon peel**
OFMW	**Organic fraction of municipal waste**
OTU	**Operational taxonomic unit**
PP	**Pineapple peel**
RBR	**Raw batch reactor**

Raw mix	**Raw mixture**
RS	**Reducing sugars**
RSBR	**Raw sequencing-batch reactor**
S/I	**Substrate-to-inoculum ratio**
SRF	**Soft-rot fungi**
SSF	**Solid-state fermentation**
SSF25d MIX	**25-day fungal-pretreated mixture**
TCD	**Thermal conductivity detector**
TCH	**Total carbohydrates**
TN	**Total nitrogen**
TS	**Total solids**
TV-H	***Trametes versicolor* hydrolysate**
VFAs	**Volatile fatty acids**
VS	**Volatile solids**
WRF	**White-rot fungi**
WS	**Wheat straw**

CHAPTER 1

1. Introduction

The consumption of fossil fuels, such as coal and petroleum-derived products, has had a significant environmental impact, as these fuels are the main contributors to air pollution due to the generation of greenhouse gases, such as carbon dioxide (CO_2), during combustion. Fossil fuels originate from finite resources, and their consumption continues to increase, currently supplying around 86% of the global energy demand. Oil, gas, and coal account for 36%, 27%, and 23% of the worldwide energy demand, respectively, whereas renewable sources represent only 14% (Abas et al., 2015), impacting air quality directly. In 2021, for instance, atmospheric CO_2 concentrations reached 417 ppm, surpassing the previous record of 407 ppm reported in 2018 (Tiseo, 2022).

As a result of the above, research on cleaner and environmentally friendly energy sources has intensified in recent years. Hydrogen (H_2) has attracted considerable interest in the biofuels field over the last decades due to its high energy potential and its clean combustion, which does not generate CO_2 emissions (Singh et al., 2015). This energy vector can be produced through different methods, such as thermochemical, electrochemical, or biological processes (Cieciura-Włoch & Borowski, 2019). Among the biological methods for hydrogen production (also called bioH_2), photofermentation and dark fermentation (DF), can be highlighted, the latter being one of the most promising methodologies for clean hydrogen production due to its simplicity, ease of scale-up, and high hydrogen yields (Chen et al., 2021). In the DF process, a degradation of organic matter into H_2, CO_2, and volatile fatty acids (VFAs) through the action of certain anaerobic microorganisms occurs. This method for H_2 production has the advantage of being able to use a wide variety of wastes as carbon sources, such as agricultural residues, wastewater, and food waste, making it especially attractive because, in addition to producing energy, it also contributes to the removal and treatment of such wastes (Bernardo Ruggeri, Tonia Tommasi, 2015; Ghimire et al., 2015; Noguer, Magdalena, et al., 2022).

However, despite the advantages that DF offers for H_2 production, some challenges still need to be addressed, particularly the inhibition of H_2 production

associated with the complexity of the substrates used. Although DF allows the valorization of carbohydrate-rich wastes, these substrates usually contain complex polysaccharide structures such as lignocellulose and starch, which are difficult for the microorganisms involved in the process to degrade. In lignocellulose waste, for instance, the presence of lignin represents one of the main barriers limiting substrate biodegradability (Mattila et al., 2017).

Carbohydrate-rich agroindustrial waste valorization is a key sustainability challenge for the development of large cities worldwide, particularly in regions where the agroindustrial sector represents an important economic activity (Ikechukwu et al., 2026). Rapid population growth and the consequent increase in demand for agricultural products lead to the generation of large amounts of waste, which can negatively affect environmental quality and human health (Duque-Acevedo et al., 2020). Sustainable waste valorization practices not only contribute to effective waste management but also enable the production of value-added products, such as biogas and volatile fatty acids. In this way, they create opportunities for both public and private sector stakeholders while promoting the recycling and valorization of materials traditionally considered industrial waste streams.

Several studies have used complex substrates such as the organic fraction of municipal waste (OFMW), lignocellulosic biomass (rice or wheat straw and corn stalks), cheese whey, and wastewater from the food industry (Show et al., 2012). Reyna-Gómez et al., 2019, for instance, used a codigestion composed of fruit peels and sewage sludge, achieving a hydrogen yield of only 25 $mLH_2 \cdot gVS^{-1}$. Similarly, Sołowski et al., 2023,evaluated hydrogen production from wheat straw and obtained a yield of 38 $mLH_2 \cdot gVS^{-1}$. Due to the above, it has been observed that the nature of the substrate used has a direct impact on H_2 production, since the more complex the substrate is, the lower the H_2 production. (Guo et al., 2014 and Monlau et al., 2012), reported that the reducing sugars (RS), present in the substrates, represent the main fraction of biomass that can be converted into H_2. Therefore, several physical, chemical, and biological pretreatment methods have been implemented to hydrolyze polysaccharides present in complex substrates such as lignocellulosic waste in order

to make them more accessible for microbial fermentation in DF, thereby increasing biogas/H_2 production (Ghimire *et al*., 2015; Hendriks & Zeeman, 2009; Mussoline *et al*., 2013; Zheng *et al*., 2014).

The complex substrates mentioned above require physical, chemical, and biological pretreatments or a combination of them to improve the degradation rate, biogas production, and H_2 yield (Ghimire et al., 2015; Hendriks & Zeeman, 2009a; Mussoline et al., 2013; Zheng et al., 2014b). Several studies have demonstrated a positive effect of substrate pretreatment on biogas production due to the hydrolysis of the lignocellulosic structure. For example, an increase of RS concentration from 2.5 μmol·mL^{-1} to 48.0 μmol·mL^{-1} was achieved using the white-rot fungus *Pleurotus ostreatus* coupled with enzymatic hydrolysis as a pretreatment method for sawdust. Likewise, a 120% increase in CH_4 yield was reported due to 33.4% delignification after the pretreatment of rice straw with *P. ostreatus* (Castoldi et al., 2014; Mackul'ak et al., 2012; Mustafa et al., 2016).

Although physical and chemical pretreatments are attractive for industrial applications due to their short processing time and effectiveness, they present the major disadvantage of requiring large amounts of energy and, in the case of chemical pretreatments, generating toxic residues that may inhibit the subsequent DF (Ghimire et al., 2015). This is where biological pretreatments gain importance for the hydrolysis of recalcitrant substrates. Complex substrates, such as lignocellulosic biomass, can be pretreated by certain fungi and actinomycetes that promote delignification and partial hydrolysis of cellulose through the secretion of enzymes (e.g., hemicellulases, cellulases, and laccases), which degrade the structural holocellulose (the hemicellulose and cellulose complex) and lignin, thereby releasing the sugars contained within the lignocellulosic matrix (Ghimire et al., 2015). Lignocellulolytic enzymes extracted from certain fungi, mainly xylanases, cellulases, and laccases, play fundamental roles in several biotechnological processes, such as biofuel production, soil and water bioremediation, as well as in the pharmaceutical industry to produce compounds of industrial interest (Carrillo-Nieves et al., 2022; Khambhaty et al., 2018).

During biological pretreatment of complex lignocellulosic substrates, a wide variety of fungal species are used to degrade the lignocellulosic structure. Brown-rot fungi (BRF), for instance, attack cellulose, whereas white-rot fungi (WRF) degrade both cellulose and lignin through the secretion of hydrolytic enzymes. Thus, WRF are widely recognized as the most efficient fungi for biomass delignification due to their unique enzymatic system, which enables efficient lignin degradation by attacking phenolic structures and ultimately converting lignin into CO_2 (Rouches et al., 2016a; Sánchez, 2009). Therefore, WRF are considered more efficient when biofuel production, such as bioethanol or biogas, is the main objective of the pretreatment process (Bhurat et al., 2020).

The biological pretreatment of substrates using fungi does not present the disadvantages associated with physical and chemical pretreatments, such as high energy consumption and the generation of toxic compounds, including furfurals and high concentrations of volatile fatty acids, which negatively affect the subsequent DF process (Kudanga & Le Roes-Hill, 2014). Therefore, the use of WRF to enhance H_2 production through the DF of lignocellulosic waste contributes to the gradual transition from fossil fuels to renewable energy sources.

There are few reports on the degradation of lignocellulosic residues using WRF for their potential implementation as substrates for H_2 production. Therefore, this research aims to study a mixture of lignocellulosic residues: fruit peels (FRP) such as banana peel (BP), melon peel (MP), and pineapple peel (PP); wheat straw (WS); and brewer's spent grain (BSG) as a substrate through biological pretreatment using the WRF *Trametes versicolor* and *Pleurotus ostreatus*, which promote delignification and partial hydrolysis of the lignocellulosic complex through the solid-state fermentation (SSF) process. The purpose is to increase the availability of RS and reduce the lignin content so that pretreated biomass can be used as a substrate in reactors to produce H_2 and other metabolites of interest via DF. Certain variables involved in the pretreatment of lignocellulosic mixture with WRF, such as pretreatment time, temperature, and fungal strain, will be evaluated using a mixed factorial design 3x2x2 with delignification and RS concentration as response

variables. In addition, during the DF process, the effect of such pretreatment on biogas and H_2 production, as well as on microbial communities and associated metabolic pathways, will be assessed.

1.1. Hypothesis

The use of white-rot fungi as a biological pretreatment method for lignocellulosic waste will promote biogas and H_2 production through the dark fermentation process.

1.2. Objectives

1.2.1. General objective

To promote the fermentation of simple sugars through a biological pretreatment with white-rot fungi on lignocellulosic substrates (FRP-WS-BSG) to enhance the H_2 production process via dark fermentation.

1.2.2. Specific objectives

- To use white rot fungi (*T. versicolor* and *P. ostreatus*) for the biological pretreatment of lignocellulosic waste mixture composed of fruit peels (FRP), wheat straw (WS), and brewers' spent grain (BSG) to promote reducing sugars fermentation.
- To determine physicochemical characteristics of the lignocellulosic mixture (TS, VS, pH, TN, and RS).
- To subject the lignocellulosic mixture to a biological pretreatment using white-rot fungi for the partial degradation of lignocellulose by adjusting variables such as temperature, treatment time, and fungal strain.
- To compare H_2 production in DF by feeding the system with the pretreated and untreated substrate (Control) and evaluate H_2 production potential using a thermally-treated microbial consortium as inoculum.
- To analyze physicochemical parameters in DF (pH, RS, TS, VS) and evaluate the production of H_2 and VFAs through gas chromatography.
- Select and enrich an efficient microbial consortium and operate DF in both batch and sequencing-batch reactors.

CHAPTER 2

2. Background

2.1. Current Status of the Energy Sector

Fossil fuels are currently the main source of energy worldwide; however, their consumption causes several environmental impacts, including the greenhouse effect, climate change, and air pollution (Zagrodnik & Sobociński, 2024). The transportation sector alone accounts for 20% of global greenhouse gas emissions (J.G.J. Olivier and J.A.H.W. Peters, 2020). Therefore, greenhouse gas emissions could be reduced by decreasing the use of conventional fuels in the transportation sector (Nordin et al., 2024).

The indiscriminate use of fossil fuels, continuous population growth, and environmental degradation compel us to establish urgent measures to meet the objectives of the United Nations 2030 Agenda for Sustainable Development, which includes 17 Sustainable Development Goals (SDGs). In this context, objective 13 is particularly relevant, as it addresses climate-related concerns. This objective can be achieved through the effective implementation of Goal 7, which emphasizes access to affordable and environmentally sustainable energy solutions (United Nations, 2015).

Global energy demand increased by 50% between 2000 and 2022, reaching 622,152,806 terajoules (TJ), with China (25.6%) and the United States (14.6%) being the largest energy consumers (IEA, 2022b). **Figure 2.1** displays the global energy sources in 2022, where oil was the predominant source (30.2%), followed by coal (27.6%) and natural gas (23.1%).

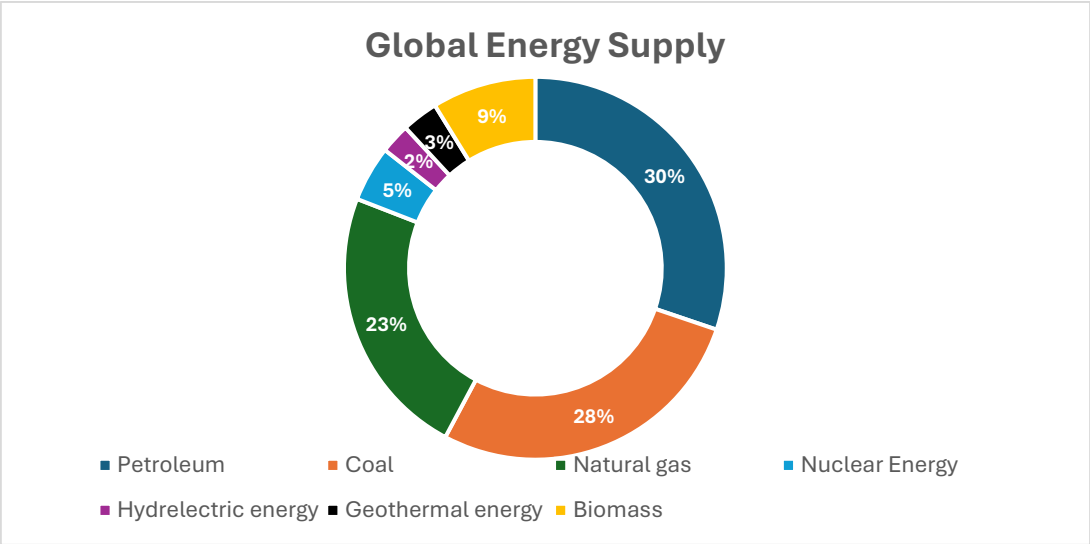


Figure 2.1. Total energy supply by source worldwide, 2022. Adapted from (IEA, 2022b).

The aforementioned is directly related to CO_2 emissions into the atmosphere. Similarly, CO_2 emissions increased by around 47% from 2000 to 2022, reaching a historic maximum in 2022 with 34,117 million tons (Mt) of CO_2 released into the atmosphere uniquely from the combustion of conventional fuels. Once again, leading the list China and the United States, accounting for 31.1% and 13.5% of total CO_2 emissions from fuel combustion, respectively (IEA, 2022a).

This gives rise to the promotion of energy transition through the use of cleaner fuels, since energy is a crucial parameter for economic growth and development. The adoption of renewable energy sources can significantly reduce greenhouse gas emissions and, consequently, mitigate environmental impacts while providing economic benefits (Melika et al., 2023). Renewable energy sources offer a sustainable alternative to fossil fuels, thereby reducing dependence on conventional energy sources.

Among the different renewable energy sources, hydroelectric energy is by far the biggest renewable energy source worldwide, followed by wind and solar energy, as can be seen in **Figure 2.2.** where the electric generation through renewable energies is shown (Hannah Ritchie, 2024).

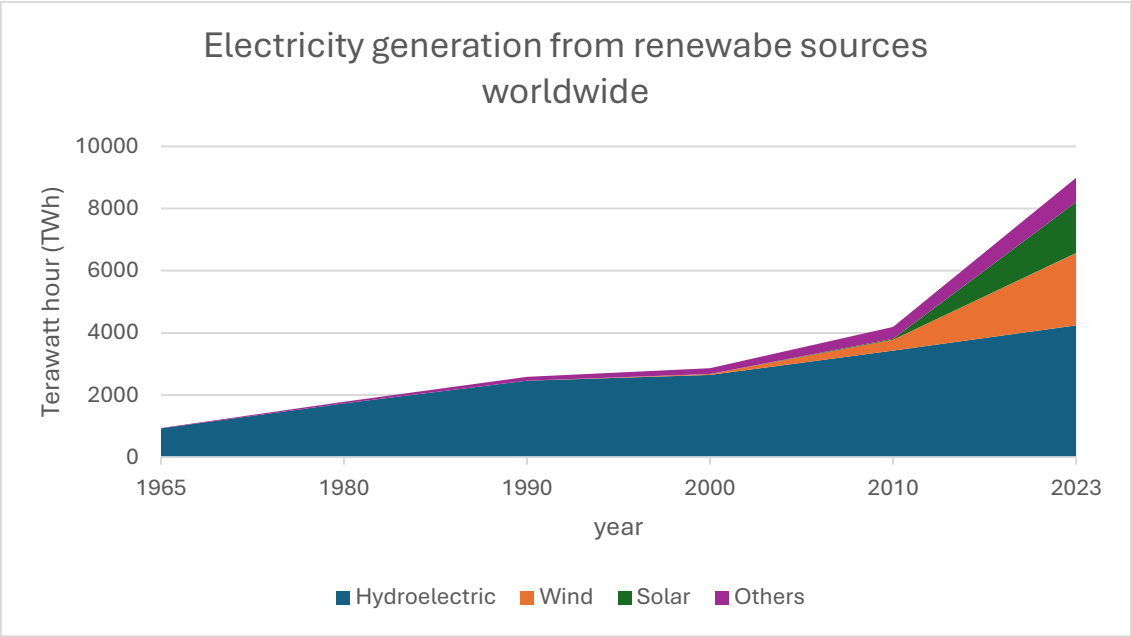


Figure 2.2. Electricity generation from renewable sources worldwide. Adapted from (Hannah Ritchie, 2024).

Regarding the use of biomass for energy production, as shown in **Figure 2.1**, it accounts for approximately 47% of the total renewable energy generated worldwide. Its importance lies in the potential to valorize agricultural, forestry, agro-industrial, and urban residues for the generation of electricity, heat, and biofuels. In France, biomass represents approximately one-third of the primary production of renewable energy (Service des données et études statistiques, 2025), whereas in Mexico, the energy recovery of urban organic waste and lignocellulosic biomass is considered a promising alternative to increase the share of renewable energy sources and promote circular economy schemes. In this context, the state of Nuevo León has emerged as a pioneer in this field, producing biogas that supplies energy to the city's metro system as well as to public parks (El Porvenir, 2022).

2.2. Hydrogen as a Promising Energy Vector

One of the most promising alternatives for the gradual replacement of fossil fuels is hydrogen (H_2), since, unlike fossil fuels, it does not emit CO_2 during combustion, releasing only water vapor. In addition, it possesses a high energy density of 120 $kJ \cdot g^{-1}$, representing approximately 2.5 times the energy density of conventional gasoline (Nazir et al., 2020).

In addition to the above, H_2 possesses a low volumetric energy density, with only 1 kg of H_2 producing an amount of energy equivalent to that generated by one gallon of gasoline (3.78 L) (Ursua et al., 2012). Energy from hydrogen is released when this gas reacts with oxygen, generating water as a byproduct, as shown in **Equation 2.1**.

$$H_2 + ½\ O_2 \rightarrow H_2O \qquad \textbf{Equation 2.1}$$

$$\Delta H = -285\ kJ \cdot mol^{-1} \text{ at } 25°C$$

$$\Delta G = -237\ kJ \cdot mol^{-1} \text{ at } 25°C$$

Hydrogen is the lightest known element and the most abundant gas in the universe, representing approximately 90% of the observable universe (Abdin et al., 2020). However, despite its abundance, obtaining elemental hydrogen from natural sources is highly challenging because naturally occurring hydrogen is too dispersed to be recovered economically, and the geological processes responsible for its generation, such as water-rock reactions involving the oxidation of Fe^{2+} to Fe^{3+} and the radiolysis of water by radioactive elements, occur over timescales ranging from thousands to millions of years. In contrast, the majority of hydrogen used today is produced industrially through steam reforming, a process that relies on fossil fuels as the primary feedstock (Ballentine et al., 2025; Nguyen et al., 2026). Consequently, the most widely used methodology to produce this promising energy vector is not environmentally sustainable (Pareek et al., 2020). Another widely used technology for commercial H_2 production is water electrolysis, including proton exchange membrane (PEM) and alkaline water electrolysis (AWE). However, these technologies require high electricity consumption, which may result in a greater environmental impact depending on the electricity source (El-Shafie, 2023).

Although hydrogen-based energy is currently expensive and still in its early stages of development and research, significant technological advances have been achieved in this field. The versatility of hydrogen lies in the fact that, in addition to the above, it can be used as a storage medium for intermittent renewable electricity and as an important energy vector in remote areas that require an electrical power supply (Abdin et al., 2020). However, some challenges must be addressed to achieve large-scale hydrogen production and enable a gradual energy transition, as current hydrogen production faces limitations associated with high energy costs, dependence on renewable electricity for processes such as electrolysis, and challenges in storage and distribution due to its low volumetric density (DAS & VEZIROGLU, 2008; Johnston et al., 2005; Singh et al., 2015).

2.3. Methodologies for hydrogen production

As mentioned above, hydrogen is currently mainly produced through the steam reforming process using fossil fuels as feedstock. However, various processes for clean hydrogen production have been developed, avoiding the use of fossil fuels.

In summary, H_2 can be produced through thermochemical, electrochemical, and biological methods. Among thermochemical methods for hydrogen production are coal gasification, steam reforming, and pyrolysis. On the other hand, the most studied electrochemical methods include water electrolysis and the use of photoelectrochemistry. Finally, biological methods for hydrogen production include water biophotolysis, photofermentation, and dark fermentation (Afanasev et al., 2024). **Table 2.1.** presents the main principles of each of these H_2 production processes, as well as the feedstock (substrate) used for this purpose.

Table 2.1. Main methodologies for hydrogen production.

Method	Process	Substrate or feedstock	Principle	Reference
Thermochemical	Steam reforming	Natural gas	It consists of reacting CH_4 with steam at high temperatures (700–1,100 °C) in the presence of a catalyst, generally Ni-based.	(LUTZ et al., 2003)
	Gasification	Coal	Coal is converted into synthesis gas (syngas) through reactions with O_2, steam, and CO_2 at high temperatures.	(X. Zhang & Lu, 2022)
	Pyrolysis	Biomass	Biomass is decomposed in the absence of O_2 at temperatures ranging from 400 to 800 °C, producing syngas, bio-oil, and biochar.	(Aminu et al., 2022)
Electrochemical	Water electrolysis	Water	It uses electricity to split water into H_2 and O_2 through an electrolyzer.	(Shiva Kumar & Himabindu, 2019)
	Photoelectro chemistry	Water	It uses sunlight to generate H_2 from water through a photoelectrochemical cell.	(Clarizia et al., 2023)
Biological	Water biophotolysis	Biomass	Photosynthetic organisms, such as algae and cyanobacteria, use sunlight to split water (H_2O) into hydrogen (H_2) and oxygen (O_2).	(Ghirardi et al., 2014)
	Photofermentation	Biomass	It uses photoheterotrophic bacteria in the presence of light and under low nitrogen concentration conditions. These microorganisms use sunlight to generate ATP and NADPH, which are subsequently employed in the reduction of protons (H^+) into H_2.	(Hallenbeck et al., 2012a)
	Dark Fermentation	Biomass, wastewater, food waste	Anaerobic process in which fermentative bacteria decompose organic substrates in the absence of light, producing H_2 as a byproduct.	(Bernardo Ruggeri, Tonia Tommasi, 2015)

The thermochemical and photochemical methods previously described in **Table 2.1**, in addition to using fossil fuels in their process (as in the case of steam reforming, gasification, and pyrolysis), require large amounts of energy and high operating temperatures, thereby affecting the environmental and economic sustainability of the process (Ishaq et al., 2022). Due to this, biological methods have emerged as an environmentally friendly alternative, since, unlike other hydrogen production methods, they require lower energy input and lower reaction temperatures, while also offering the advantage of using recalcitrant lignocellulosic residues (agroindustrial waste, forestry residues, and municipal solid waste), wastewater, and food waste as substrates (Bundhoo & Mohee, 2016). In particular, dark fermentation has gained increasing attention because it couples H_2 production with the treatment of organic wastes and wastewater, promoting both renewable energy generation and environmental remediation (Alqahtani, 2024; Qu et al., 2022).

2.4. Hydrogen and Biogas from Lignocellulosic Biomass

In recent decades, there has been a growing interest in lignocellulosic biomass as an energy source, since different types of biofuels can be produced from it, including bioethanol, biodiesel, and biogas (including CH_4 and H_2), which represent important alternative sources for clean energy production (Kumara Behera & Varma, 2016). Biomass is defined as all organic matter derived from plants and/or animals that is intended to be used as a storage source of chemical energy to provide heat, energy, or transportation fuels (Bernardo Ruggeri, Tonia Tommasi, 2015). Therefore, there is a wide variety of biomass that can be used for this purpose, ranging from the biodegradable fraction of residues generated from agricultural activities to the organic fraction of industrial and municipal waste.

Lignocellulose is mainly composed of three fundamental compounds: cellulose, hemicellulose, and lignin (**Figure 2.3**). Cellulose is the most abundant biopolymer on Earth and consists of a glucose polysaccharide linked through β (1-4) glycosidic bonds. These bonds provide cellulose with high resistance to hydrolysis. The second most abundant compound in lignocellulosic biomass is hemicellulose, which is an amorphous heteropolymer composed of various sugars, including mannose, xylose, and glucose, among others. Due to its amorphous structure, hemicellulose is more

susceptible to hydrolysis compared to cellulose. On the other hand, lignin, the third major component of lignocellulose, is composed of a variety of aromatic alcohols and is intertwined with the cellulose and hemicellulose fraction (holocellulose) within the biomass structure. Therefore, lignin contributes to the rigidity of lignocellulosic materials, such as trunks, stems, and fruit peels, for example (Williams et al., 2017).

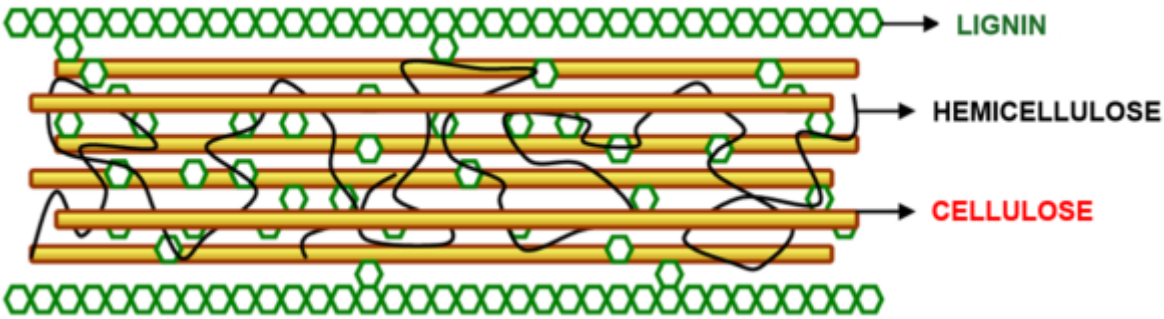


Figure 2.3. Simplified structure of lignocellulose. Obtained from (Mussatto M. I. & Teixeira J. A., 2010)

The holocellulose complex in lignocellulose, that is the fraction containing sugars, can be utilized by certain microorganisms to produce biofuels and other compounds generated during the bioconversion process. Biogas production from biomass, for example, occurs through two fundamental steps: the depolymerization of holocellulose into fermentable sugars and the fermentation of these sugars into H_2, CO_2, and volatile fatty acids (VFAs) as byproducts (Tursi, 2019; Williams et al., 2017).

One of the most promising methodologies for biogas and biological hydrogen production is dark fermentation (DF). This process, unlike photofermentation and water biophotolysis, does not require an external light source, is economically feasible, and has the versatility to utilize various types of substrates, including lignocellulosic biomass (Cieciura-Włoch & Borowski, 2019).

Various types of lignocellulosic biomass have been studied for H_2 production. Rice, corn, and wheat residues, for instance, have been used as substrates in the DF process, achieving H_2 production ranging from 12 to 7,019 $mLH_2 \cdot L^{-1}$ (Soares et al., 2020). In a study conducted by He et al., 2014, for example, H_2 production was evaluated using rice straw as substrate, achieving a yield of 28 $mLH_2 \cdot gVS^{-1}$. On the

other hand, a study carried out by T. Zhang et al., 2020, in which corn stover was used as substrate for hydrogen production, achieved a yield of 36.08 $mLH_2 \cdot g\ biomass^{-1}$. Likewise, mixtures of lignocellulosic residues have also been implemented for this purpose, such as in the study conducted by (Reyna-Gómez et al., 2021), where a mixture of fruit peels (melon, pineapple, and papaya) was used as substrate for hydrogen production in a hybrid upflow anaerobic reactor (HUAR), achieving a maximum yield of 650 ± 50 $mLH_2 \cdot gCOD\ removed^{-1}$.

The above reaffirms the importance of using lignocellulosic residues for biogas and hydrogen production, thereby contributing not only to the generation of clean energy but also to the management and valorization of waste.

2.5. Fundamentals of Dark Fermentation and Its Importance as a Sustainable Method for Clean Hydrogen Production

DF, as described above, has great versatility in substrate utilization, is easy to scale up, and does not require an external light source, unlike photofermentation. Additionally, it presents high biogas and hydrogen yields (K. F. Adekunle & Okolie, 2015). This process involves the transformation of a wide range of organic substrates, including lignocellulosic biomass, into a mixture of products such as acetic acid, butyric acid, and hydrogen. In summary, this bioprocess occurs in the absence of O_2 and light (Bastidas-Oyanedel et al., 2015). During DF, simple sugars such as glucose and xylose serve as the main carbon sources for hydrogen-producing bacteria (HPB). However, some polysaccharides, such as starch and cellulose, also possess the capacity to supply the carbon demand of the microorganisms involved in the process. Nevertheless, due to their structural complexity, these carbohydrates are more difficult to process (Ntaikou et al., 2010).

Dark fermentation is essentially part of anaerobic digestion (AD), comprising the first three stages of the latter process (**Figure 2.4**), excluding methanogenesis (CH_4 production from H_2 and other byproducts) (Bernardo Ruggeri, Tonia Tommasi, 2015). In the first stage of DF, hydrolysis, high-molecular-weight compounds such as lipids, polysaccharides, and proteins are transformed into simpler soluble compounds, including monosaccharides, amino acids, and other organic molecules. In the

second stage, acidogenesis, monosaccharides, fatty acids, and amino acids produced during hydrolysis are converted into organic acids, alcohols, CO_2, and H_2. Finally, during the third stage of DF, acetogenesis, the volatile fatty acids (VFAs) produced during the process with carbon chains longer than one carbon unit are oxidized into acetate, CO_2, and H_2 (K. F. Adekunle & Okolie, 2015; Brémond et al., 2018a).

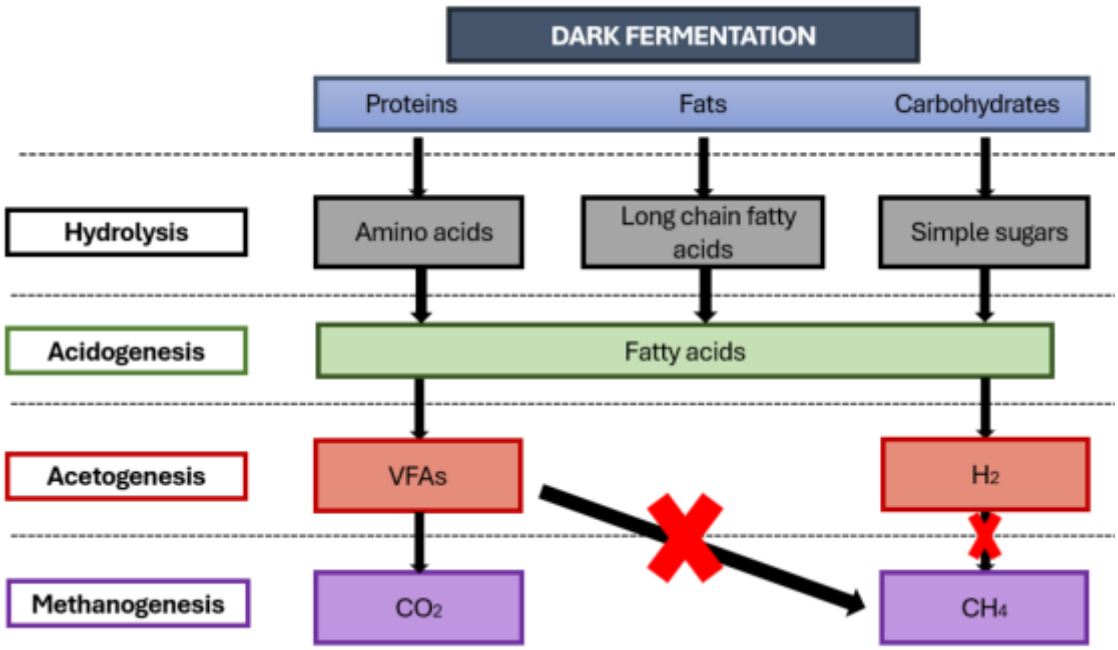


Figure 2.4. General scheme of the different stages in the dark fermentation process. Adapted from (Bernardo Ruggeri, Tonia Tommasi, 2015).

Regarding microorganisms involved in DF, in general, mixed cultures containing acetogenic and acidogenic bacteria are generally employed, since these microorganisms can produce H_2 through their metabolism. Commonly, the inoculum used in the process consists of sludge from anaerobic digesters, activated sludge, compost, ruminant manure, and river sediments. However, the use of microbial consortia may pose a risk to the process, as hydrogen-consuming microorganisms as well as non-producing microorganisms may also be present (Tapia-Venegas, Ramirez-Morales, et al., 2015). Hydrogen consumers include hydrogenotrophic

methanogenic archaea, homoacetogenic bacteria, and nitrate- and sulfate-reducing bacteria, which use electrons from hydrogen to reduce specific substrates (Tapia-Venegas, Cabrol, et al., 2015; Wang et al., 2013). Nevertheless, since these microorganisms are unable to form spores, unlike many hydrogen-producing bacteria, certain pretreatments can be applied to the inoculum to inactivate microorganisms detrimental to the process, such as acid or alkaline treatments, heat treatments, or by decreasing the hydraulic retention time (HRT) in a continuous system (Castelló et al., 2020; Ntaikou et al., 2010; Tapia-Venegas, Cabrol, et al., 2015).

On the other hand, HPB can be classified as spore-forming and non-spore-forming, or as strict anaerobes and facultative anaerobes. Two of the most important genera of hydrogen-producing bacteria are *Clostridium* and *Enterobacter*, which are strict anaerobic spore-forming bacteria and facultative anaerobic non-spore-forming bacteria, respectively (Tapia-Venegas, Cabrol, et al., 2015).

H_2 production is not limited solely to the metabolism of *Clostridium* and *Enterobacter*. In a study conducted by García-Depraect et al., 2017, for instance, bacteria belonging to the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Clostridium* were reported as hydrogen producers. Likewise, *Prevotella* and *Bacillus* have also been identified as HPB in other studies (Castelló et al., 2020).

There are certain differences in H_2 production between facultative anaerobic and strict anaerobic microorganisms. The former can produce a maximum of 2 moles of H_2 per mole of glucose consumed, whereas strict anaerobes, such as *Clostridium*, can produce up to 4 moles of hydrogen per mole of glucose consumed, making them more efficient for H_2 production (Bernardo Ruggeri, Tonia Tommasi, 2015).

Figure 2.5 displays the two main metabolic pathways involved in H_2 production through DF from glucose. Depending on the microorganisms involved and their metabolism, pyruvate can be converted into acetyl-CoA and CO_2, generating a reduced ferredoxin molecule (Fd_{red}), which can subsequently be reoxidized through hydrogen production.

Another possibility is the conversion of pyruvate into acetyl-CoA and formate. In the first pathway, mainly used by strict anaerobic bacteria such as *Clostridium*, the reaction is catalyzed by pyruvate:ferredoxin oxidoreductase (PFOR). The second pathway depends on the presence of formate hydrogen lyase (FHL) and is, in contrast, carried out by facultative anaerobic bacteria (Cai et al., 2011; Tapia-Venegas, Ramirez-Morales, et al., 2015).

During the conventional hydrogen production carried out by microorganisms with an active FHL pathway, the degraded formate is converted into H_2 and CO_2 through the catalysis of formate hydrogen lyase. Depending on the microorganism involved, this reaction may occur through either a [NiFe]-hydrogenase or a formate-dependent [FeFe]-hydrogenase. Subsequently, acetyl-CoA is oxidized to acetate, with the production of one ATP molecule. In these cases, microorganisms are unable to access the NADH produced during glycolysis to generate additional hydrogen (H_2). Therefore, NADH is oxidized through the production of various reduced carbon compounds (such as ethanol or lactate), which limits the yield to a maximum of 2 moles of H_2 per mole of glucose (Hallenbeck et al., 2012b).

Hydrogen production in microorganisms through the PFOR pathway occurs via the oxidation of reduced ferredoxin (Fd_{red}) by a ferredoxin-dependent hydrogenase (Fd-[FeFe]). In addition, under specific conditions, it is possible to reoxidize the NADH generated during glycolysis to produce additional hydrogen molecules through two other hydrogenases: one NADH-dependent hydrogenase (NADH-[FeFe]) and another dependent on both NADH and Fd_{red} (NADH-Fd_{red}-[FeFe]).

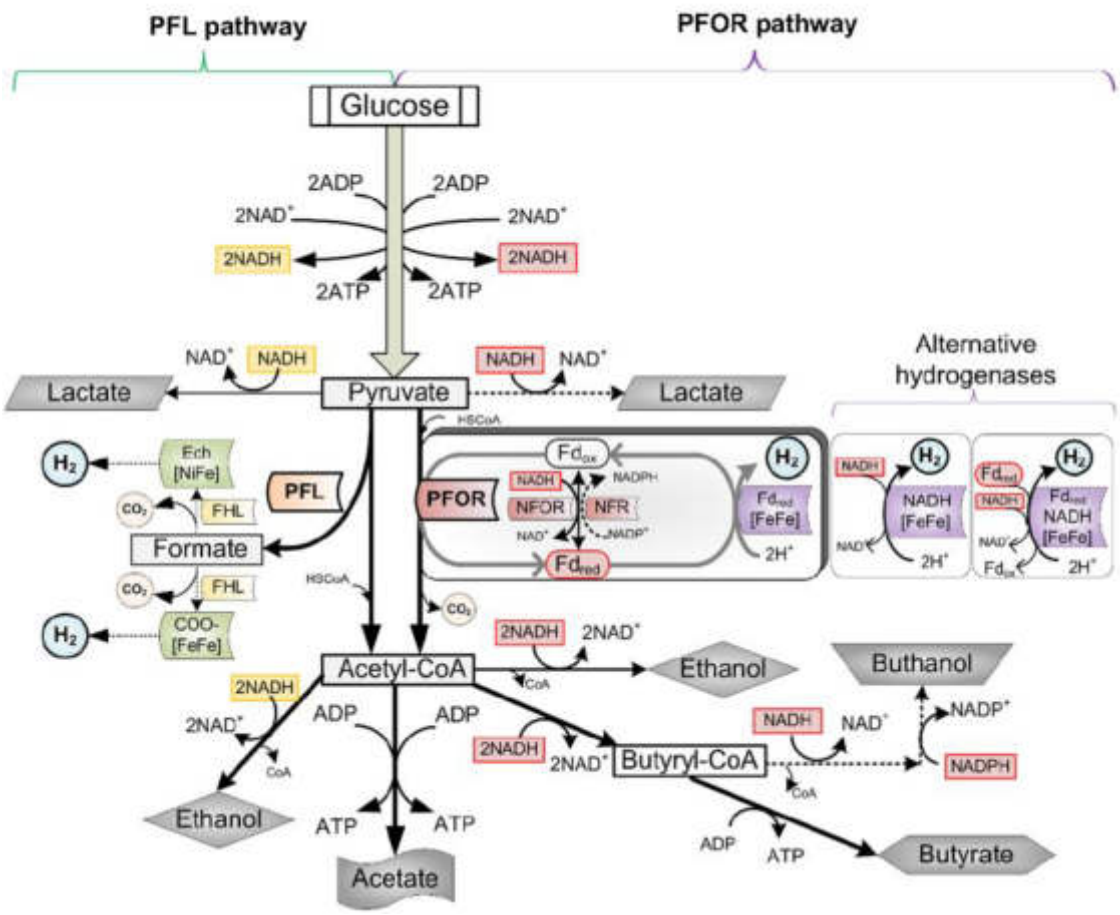


Figure 2.5. Metabolic pathways involved in H_2 production through the DF process. Adapted from (Tapia-Venegas, Ramirez-Morales, et al., 2015).

DF, being a complex system in which diverse microorganisms are involved in the production of H_2 and VFAs, presents competitive yields and is more economical than other biological methodologies for hydrogen production. However, it also faces some challenges, especially when complex substrates such as lignocellulosic biomass are used.

2.6. Challenges in H_2 production Through Dark Fermentation Using Lignocellulosic Biomass

As mentioned above, dark fermentation produces a mixture of VFAs, solvents, and gases, whose proportions vary depending on the microorganisms involved and their metabolic pathways (Cai et al., 2011). Therefore, if an efficient system for H_2 production is desired, several process parameters must be considered, since the

process is affected by diverse factors such as inoculum pretreatment (to inhibit hydrogen-consuming microorganisms), pH, temperature, hydrogen partial pressure, bioreactor configuration, and, particularly when lignocellulosic biomass such as agroindustrial waste is used as a substrate, the pretreatment applied to the substrate (Bastidas-Oyanedel et al., 2015).

The pretreatment of agroindustrial waste is essential for breaking down its main components (cellulose, hemicellulose, and lignin) and efficiently converting them into bioenergy. An effective pretreatment process must consider several aspects, such as energy consumption, economic feasibility, reduction of biomass particle size to increase the surface area available for hydrolysis, avoidance of inhibitory compound production, and reduction of the crystallinity index of lignin and cellulose (Mankar et al., 2021).

The biomass composition can vary depending on the source. This implies the need to develop pretreatment methods adapted to their specific characteristics. The available approaches are divided into physical, chemical, and biological methods, each with its own advantages and disadvantages, and the success of their application depends on the optimization of these factors to maximize process efficiency and sustainability

2.7. Pretreatment methods

There are various pretreatment methods aimed at partially hydrolyzing the lignocellulosic structure and improving its accessibility during the subsequent biofuel production process, whether for biogas, H_2, or even CH_4 production (Hendriks & Zeeman, 2009a). Pretreatment methods are classified as physical, chemical, and biological, and they can be applied either individually or together (Kainthola et al., 2021).

2.7.1. Physical pretreatments

Physical pretreatments are based on those methods that do not use chemical reagents or microorganisms, with milling or grinding, hydrothermal hydrolysis, and ultrasound or microwave irradiation being the most used physical pretreatment methods (Zheng et al., 2014).

Lignocellulosic biomass milling or grinding is used to decrease the particle size and is usually combined with other pretreatment methods to improve the efficiency in biofuel production. By reducing the lignocellulosic particle size, the accessible surface area increases, the degree of cellulose crystallinity decreases, and the degree of cellulose polymerization is reduced, thereby improving its accessibility and degradability (Kratky & Jirout, 2011; Zheng et al., 2014). This has demonstrated a significant improvement in biofuel production when particle size is reduced. For instance, De la Rubia et al., 2011 improved methane production yields by reducing the particle size of bagasse and coconut fiber from 5 mm to 0.85 mm, while Sharma et al., 1988 increased biogas production by reducing the particle size of forest residues from 30 mm to 0.088 mm. However, the milling and grinding of lignocellulosic biomass are considered among the most expensive pretreatment methods due to the high energy demand required, making them less attractive at an industrial scale (Zheng et al., 2014).

Hydrothermal hydrolysis is another of the most widely used physical methods for the pretreatment of lignocellulosic biomass. In this method, the biomass is heated under high pressure and saturated with steam for a short period of time, after which the pressure is rapidly reduced to terminate the reaction, causing the biomass to undergo explosive decompression. Conditions of 160–260 °C, 0.69–4.83 MPa, and various reaction times are commonly employed (Sun & Cheng, 2002). This pretreatment method has proven to be effective, showing an increase in methane yield from wheat straw of approximately 20–30% compared to untreated wheat straw (Bauer et al., 2010). However, hydrothermal hydrolysis may cause excessive degradation of hemicellulose and/or cellulose, and it also promotes the generation of inhibitory compounds such as furfurals and hydroxymethylfurfurals, thereby limiting the efficiency of this method.

Finally, irradiation-based hydrolysis is another physical pretreatment commonly employed for lignocellulosic residues, including microwave, ultrasound, and gamma-ray irradiation. Among these methods, microwave irradiation is the most extensively studied, in which energy is generated through an electromagnetic field, directly

irradiating the material and promoting a rapid increase in temperature (Zheng et al., 2014). For example, Jackowiak et al., 2011 used microwave irradiation at 150 °C to pretreat wheat straw, observing a 28% improvement in methane yield. However, other studies have evaluated the effect of this pretreatment on improving the biodegradability of lignocellulosic substrates for biogas production, without observing any significant improvement, depending on the biomass composition studied. Therefore, some authors have reported that microwave pretreatment could be combined with another pretreatment method, such as chemical hydrolysis (Bichot et al., 2021).

On the other hand, ultrasound has also been employed as a pretreatment method for lignocellulosic biomass. In summary, this method is based on increasing the surface area and reducing the degree of lignocellulosic polymerization through the generation of cavitation, resulting in physical and chemical changes in the biomass structure and leading to cell wall disruption (Cesaro et al., 2012; Kumakura & Kaetsu, 1983), Recently, ultrasound pretreatment has been reported to improve biogas production yields by 24–34% (Cesaro et al., 2012). However, these methods are usually expensive, as physical pretreatments, in general, demand high energy and present difficulties when handling large volumes of biomass, thereby limiting their scalability and industrial application (Hendriks & Zeeman, 2009a).

2.7.2. Chemical pretreatments

Chemical pretreatments for lignocellulosic biomass have been widely used, and their classification depends on the reagent utilized, including acids, alkalis, and organic solvents. Acid pretreatment breaks glycosidic bonds and mainly solubilizes hemicellulose and part of the lignin, thereby making cellulose more accessible for subsequent enzymatic hydrolysis. The most used acids are phosphoric, nitric, hydrochloric, and sulfuric acids, with the most important process parameters being reaction time, acid concentration, temperature, and solid-to-liquid ratio. However, the use of concentrated acids has the disadvantage of transforming hemicellulosic sugars into toxic compounds such as acetic acid, furfural, and 5-(hydroxymethyl)furfural (HMF), which may inhibit microbial growth in the hydrolysate

if a subsequent fermentation process is carried out. Therefore, the use of diluted acids is generally preferred (Lorenci Woiciechowski et al., 2020).

Alkaline pretreatment removes acetyl groups, helps to solubilize lignin and hemicellulose, and also reduces the crystallinity of cellulose. This pretreatment is widely used in the paper industry for cellulose recovery (Lorenci Woiciechowski et al., 2020). Commonly used alkaline agents include sodium hydroxide, sodium carbonate, calcium hydroxide, and hydrogen peroxide. The effect of alkaline pretreatment is greater compared to acid pretreatment because it reduces lignin, which is the main inhibitor of hydrolysis. However, one disadvantage is that it requires long reaction times, as well as a neutralization step after the treatment (H. Zhang et al., 2021).

In organic solvent pretreatment, either pure solvents or their aqueous solutions (such as acetone, glycerol, ionic liquids, ethanol, among others) can be employed. As a result, high-purity cellulose is obtained, decreasing lignin recalcitrance and increasing the surface area of cellulose (K. Zhang et al., 2016). Although this type of pretreatment has the advantage of relatively low toxicity, its main disadvantage lies in its high cost (H. Zhang et al., 2021).

2.7.3. Biological pretreatments

Biological pretreatments of substrates refer to the use of enzymes produced by microorganisms to hydrolyze hemicellulose structure, or the use of certain organisms known for their capacity to secrete a wide variety of enzymes that degrade lignocellulose.

Enzymatic pretreatments consist of using specific enzymes such as cellulases or hemicellulases to enhance the hydrolytic phase of DF or AD. There are four ways to use enzymes for lignocellulosic biomass hydrolysis: (1) before the fermentation process for biogas production, in a separate reactor; (2) directly in the bioreactor for biogas production; (3) directly in the hydrolysis and acidification reactor (two-phase process); and (4) in the recirculated effluent from dark fermentation or anaerobic digestion (Brémond et al., 2018b).

The most studied enzymes in this field are those that modify the lignin structure, since the main challenge of using lignocellulosic biomass for biogas production lies in the bond existing between lignin and cellulose/hemicellulose (Monlau et al., 2013). However, enzymes such as cellulases, hemicellulases, amylases, and pectinases have also been employed, as they hydrolyze carbohydrates present in holocellulose (Brémond et al., 2018b). Enzymatic pretreatment has shown promising results by increasing biogas, hydrogen, and methane yields at laboratory scale. However, at larger scales, it becomes less feasible due to the high cost of enzymes and the reduction in enzymatic activity after their addition to the system (Brémond et al., 2018b).

Another biological pretreatment applied for the partial hydrolysis of lignocellulosic biomass, as mentioned above, is the use of certain microorganisms to partially hydrolyze the lignocellulosic structure. This approach does not require high temperatures and does not involve the use of acids, bases, or any other chemicals. In addition, it requires much less energy to be carried out and does not produce harmful byproducts during the hydrolysis phase, thereby overcoming the disadvantages presented by physical and chemical pretreatment methods. Furthermore, unlike enzymatic pretreatments, these methods are less expensive, making them more attractive for larger-scale applications (Nadir et al., 2019). However, the main disadvantage is the time required for this pretreatment, which can range from one week to up to 90 days, depending on the case (Ravindran & Jaiswal, 2016; Wan & Li, 2012a).

Biological pretreatments using microorganisms can be classified into bacterial and fungal treatments. The microorganisms most used for the pretreatment of lignocellulosic biomass are filamentous fungi, which can be easily found in the environment (soil, plants, and lignocellulosic residues). Lignocellulose-degrading fungi are classified into three groups: white-rot fungi, brown-rot fungi, and soft-rot fungi (Bari et al., 2016; Chaturvedi & Verma, 2013). Among these organisms, white-rot fungi are considered the most effective due to their ability to degrade the lignin located on the surface of holocellulose (cellulose and hemicellulose), whereas

brown-rot and soft-rot fungi degrade only a minimal fraction of lignin (Nadir et al., 2019).

Before focusing on white-rot fungi pretreatment, as a conclusion of this subsection 2.7, **Table 2.2** presents the advantages and disadvantages of each pretreatment method.

Table 2.2. Main advantages and disadvantages of each pretreatment method. Adapted from (Nadir et al., 2019).

	Physical	Chemical	Biological
Objective	Reduce particle size, increase the surface area, and reduce cellulose crystallinity	Hydrolyze lignin, cellulose, and hemicellulose	Degrade lignin from holocellulose components
Type	• Milling • Grinding • Freezing • Radiation	• Acid • Alkaline	• Microbial consortia • Fungi • Enzymes
Advantages	• Low environmental impact • High effectiveness • Short pretreatment time	• Less dangerous process condition • Lack of byproducts degradation	• Environmentally friendly • Does not require chemical reagents • Low energy consumption
Disadvantages	• High energy requirement • High cost	• Toxicity • Corrosiveness of equipment • Inhibitory compounds generation	• Longer pretreatment times • Need continuous monitoring of microorganism growth

2.8. White-Rot Fungi as a Pretreatment Method for Lignocellulosic Biomass

As previously mentioned, there are three distinct groups of rot fungi used for the partial hydrolysis of lignocellulose: brown-, soft-, and white-rot fungi.

In the case of brown-rot fungi (BRF), they extensively degrade the polysaccharides present in the lignocellulosic structure but are only capable of slightly modifying the lignin structure. Due to their affinity for degrading holocellulose, the relative lignin content increases. However, despite this, the recalcitrance of

lignocellulose decreases and enzymatic digestibility may improve, probably due to the increase in structural porosity and the reduction in cellulose crystallinity, as well as its degree of polymerization. Most brown-rot fungi degrade cellulose to such an extent that the improvement in enzymatic digestibility cannot compensate for the total sugar loss during the pretreatment process (Vasco-Correa et al., 2016). Examples of the most studied brown-rot fungi for lignocellulosic biomass pretreatment include *Coniophora puteana* and *Postia placenta*, which have been used, for example, to pretreat softwood (pine), achieving sugar yields of approximately 20% after 35 days of pretreatment (Ray et al., 2010).

On the other hand, soft-rot fungi (SRF) are another group of fungi that have been studied for their potential to degrade lignin in lignocellulosic biomass. These fungi, similarly to brown-rot fungi, degrade holocellulose, and some of them are capable of altering the lignin structure and mineralizing it. However, despite this capability, their ligninolytic enzyme system is still not sufficiently understood. Therefore, there are relatively few studies reporting their use as a pretreatment for lignocellulosic residues in biofuel production. For example, the fungus *Mycothecium roridum* showed 50% delignification of the plant *Parthenium* sp. after 7 days of pretreatment, while leaving the cellulose fraction intact (Tiwari et al., 2013).

Finally, white-rot fungi (WRF) are the organisms most extensively studied for the pretreatment of lignocellulosic biomass due to their high lignin degradation capacity and their intrinsic ability to mineralize it into CO_2 and water. WRF are a diverse group of basidiomycetes capable of degrading the three main components of lignocellulose, producing a white and fibrous degradation pattern (hence their name). Within this group of fungi, two different degradation patterns can be identified: selective and non-selective degradation. Selective WRF preferentially degrades lignin, whereas non-selective white-rot fungi simultaneously degrade the three components of lignocellulose (Vasco-Correa et al., 2016).

Evidently, if the objective of pretreatment is to improve biofuel production (biogas, H_2, or CH_4), selective degraders are preferred as a pretreatment method, since holocellulose is the primary substrate to produce biofuels and other byproducts in

the context of biorefineries (Vasco-Correa et al., 2016; Wan & Li, 2012b). One of the most extensively studied WRF is *Phanerochaete chrysosporium*, as it possesses a powerful ligninolytic system that follows a simultaneous polysaccharide degradation pathway, making it not the most effective fungus for lignocellulosic biomass pretreatment aimed at biofuel production. Other fungi widely studied for this purpose include *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Irpex lacteus*, and *Trametes versicolor* (Vasco-Correa et al., 2016). The following section describes the enzymatic mechanisms of WRF acting on lignocellulosic biomass and how they improve its accessibility to enhance H_2 and CH_4 production and yields

2.9. Degradation Mechanisms of White-Rot Fungi for Lignocellulose Hydrolysis

WRF, in addition to degrading holocellulose polysaccharides through their hydrolytic system, possess a secondary metabolic process that enables them to depolymerize the lignin fraction (Paliwal et al., 2012). These fungi are more frequently found in hardwood and preferentially degrade the S units of lignin (Vasco-Correa et al., 2016).

Therefore, unlike brown-rot or soft-rot fungi, white-rot fungi possess two extracellular enzymatic systems: the hydrolytic system and the ligninolytic system. The hydrolytic system promotes the hydrolysis of polysaccharides present in holocellulose through the secretion of enzymes such as cellulases and hemicellulases, which depolymerize polysaccharides into simple sugars (Andlar et al., 2018). In contrast, the ligninolytic system is mainly composed of three enzymes that catalyze lignin oxidation: lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases. However, not all WRF produce these three enzymes; they may produce either one of them, a combination of them, or, in the best-case scenario, all three enzymes simultaneously (Rouches et al., 2016; Tuor et al., 1995).

In addition to these enzymes, other peroxidases have also been reported to participate in lignin degradation, including versatile peroxidases (VP), manganese-independent peroxidases (MiP), and dye-decolorizing peroxidases (DyP) (Rouches et al., 2016).

As previously mentioned, lignin is a polymer formed through the oxidative polymerization of alcohols such as p-coumaryl, coniferyl, and sinapyl alcohols, which are the precursors of the H, G, and S units of lignin. This lignin complex is highly inaccessible to enzymes, which explains its recalcitrance. Although lignin monomers are phenolic compounds, 80–90% of its structure is non-phenolic because, during polymerization, most of the linkages involve the phenolic position (C4 ether), with the β-O-4 ether bond being the most common. However, the α-carbon can also form ether linkages.

C–C bonds are mainly formed between the β-carbon and other carbons, such as the 1-, 5-, or another β-carbon, or between the 5-carbon of each monolignol. These types of bonds cannot be easily hydrolyzed. In fact, the only microorganisms capable of hydrolyzing the lignin complex into CO_2 and water are WRF through oxidative reactions catalyzed by the enzymes of the ligninolytic complex (Reid, 1995; Vasco-Correa et al., 2016).

Among the main enzymes produced by the ligninolytic system, laccases and MnPs act predominantly on the phenolic compounds of lignin, whereas LiPs mainly act on non-phenolic compounds, which constitute most of the lignin structure. However, under certain conditions, MnPs and laccases may also act on non-phenolic regions. These three enzymes carry out a one-electron oxidation process. On the other hand, versatile peroxidases (VPs) can degrade both phenolic and non-phenolic compounds of lignin (Hatakka, 1994; Rouches et al., 2016; Wan & Li, 2012c).

As previously mentioned, LiPs mainly act on the non-phenolic compounds of lignin, generating cation radicals due to their high redox potential, which break the α-β carbon bonds and act as mediators in ring-opening reactions. These enzymes are not produced by all white-rot fungi; however, fungi incapable of secreting them still exhibit a high ligninolytic capacity due to the secretion of other enzymes (Furukawa et al., 2014).

On the other hand, MnPs are the most common ligninolytic enzymes secreted by white-rot fungi (Floudas et al., 2012; Hatakka, 2001). MnPs convert Mn^{2+} into Mn^{3+}.

This chelated Mn^{3+} oxidizes phenolic compounds due to its low redox potential, unlike LiPs. However, in the presence of unsaturated lipids, for example, MnPs can use peroxide radicals as mediators to oxidize the non-phenolic complexes of lignin (Youn et al., 1995).

Laccases, for instance, possess four fully oxidized copper ions in their active site. They catalyze lignin oxidation one electron at a time. The complete catalytic cycle transfers four electrons to the four Cu ions. Laccases can oxidize the phenolic complex by forming a phenoxy radical; however, they cannot directly oxidize the non-phenolic fraction of lignin due to their low redox potential.

For this purpose, laccases can oxidize low-molecular-weight mediators, which act as electron carriers and diffuse into the insoluble lignin structure to oxidize it. In this way, they are able to depolymerize both the phenolic and non-phenolic fractions of lignin. In fact, some WRF produce laccases as their only ligninolytic enzyme (Wan & Li, 2012c; Youn et al., 1995).

The enzymatic delignification promoted by white-rot fungi varies depending on the type of fungus used, the substrate to be pretreated, and even the cultivation conditions during the pretreatment phase, which can be implemented under either liquid or solid conditions. Solid cultures are referred to as solid-state fermentation (SSF), a bioprocess recognized as the optimal approach for cultivating white-rot fungi and promoting the highest delignification of lignocellulosic substrates. Several studies have demonstrated that ligninolytic activity is greater in SSF than in liquid cultures, attributing this phenomenon to the fact that the conditions involved in SSF are more similar to those found in the natural environment (Hendriks & Zeeman, 2009b)

In addition, the system designs involved in SSF are simpler and less expensive compared to those used in liquid cultures, while also being more resistant to microbial contamination because of the lower moisture content present in SSF systems (Rouches et al., 2016).

2.10. State of the art in the Use of White-Rot Fungi to Enhance Biogas Production

Thanks to their important enzymatic capabilities for hydrolyzing the lignocellulosic structure, WRF have been widely employed as a pretreatment method for bioethanol production. However, the use of WRF as a pretreatment method for biogas production (namely H_2 or CH_4) remains largely unexplored.

Due to the increase in lignocellulosic biomass porosity caused by the action of WRF, together with the depolymerization of biomass compounds, substrate accessibility increases, thereby improving H_2 yields (Yan et al., 2022). Among the most studied WRF species for this purpose are *Phanerochaete chrysosporium, Trametes versicolor, Trametes hirsuta, Ceriporiopsis subvermispora, Pleurotus ostreatus, Trichoderma harzianum y Phanerochaete chrysosporium* (C. L. Cheng et al., 2011).

In a work conducted by Garduño et al., 2024, for instance, a 25% increase in H_2 yield was observed after using brewery waste sludge pretreated with *Pleurotus ostreatus* for 12 days. Similarly, Mustafa et al., 2017, reported a positive impact on CH_4 yield using rice straw pretreated with *Pleurotus ostreatus* and *Trichoderma reesei*, obtaining yields of 263 $L \cdot kgVS^{-1}$ (a 120% increase compared to the control) and 214 $L \cdot kgVS^{-1}$ (a 78.3% increase compared to the control), respectively.

This improvement in gaseous biofuel yields is mainly related to the increased accessibility of sugars present in the holocellulose fraction of lignocellulose, since fungal pretreatment depolymerizes lignin, thereby enhancing the hydrolysis stage of DF and AD processes for H_2 and CH_4 production, respectively, ultimately improving the efficiency of these processes. Table 2.3 presents selected studies on fungal pretreatment of lignocellulosic biomass for improving performance in DF and AD.

Table 2.3. Background on the use of fungi to improve dark fermentation/anaerobic digestion processes.

Substrate	Fungal strain	Fermentation parameters	Biogas, CH_4 o H_2 improvements	Reference
Rice straw	*Phanerochaete chrysosporium*	Batch in mesophilic conditions for 35 d	35 % more biogas	(Phutela, 2011)
Brewers' wastewater	*Pleurotus ostreatus*	Batch 120 mL for 30 h at 35°C, pH 6.4.	25 % increase in H_2 yield	(Garduño et al., 2024)
Food waste	*Pleurotus djamor*	4 L WV, 40°C, 50-90 RPM	3.8-fold higher H_2 yield than the control group	(Bhurat et al., 2023)
Corn stover	*Trichoderma reesei*	Batch in thermophilic conditions for 4 d	209% increase in cumulative H_2 production	(X. Y. Cheng & Liu, 2012)
Castanea sativa leaves	*Auricularia auricula-judae*	Continuous at mesophilic conditions	15 % more CH_4 volume	(Mackuľak et al., 2012)
Rice straw	*Phanerochaete chrysosporium*	4 L, 63 d, 30°C, pH 7-8	34.73 % more biogas and 46.19 % more CH_4	(Ghosh & Bhattacharyya, 1999)
Wheat straw	*Phanerochaete chrysosporium* + *cellulase of Trichoderma atrviridae*	40°C, pH 6.0-6.5, 14 h, 120 RPM.	1.8-fold higher H_2 than the untreated group.	(Zhi & Wang, 2014)
Oat straw	*Trichoderma viride and Phanerochaete chrysosporium*	60 mL, 65°C, 90 RPM	Improvement in H_2 production of 165%	(Zhou & Li, 2016)

CHAPTER 3

3. Materials and methods

The experimental work presented in this research was carried out in two different places: Monterrey, Mexico, and Narbonne, France.

The experimental stages involving the evaluation of substrate pretreatment by SSF and the BHP test were conducted at the facilities of the Universidad Autónoma de Nuevo León (UANL) in Monterrey, Mexico. On the other hand, the experimental stages comprising the modified BHP test, the BMP test, and process scale-up in batch and sequential batch reactors were performed at the Laboratoire de Biotechnologie de l'Environnement (LBE) of INRAE in Narbonne, France.

As a result, certain characterization techniques, the inoculum source, and the physicochemical characteristics of the individual substrates presented in this study differ among the experimental stages. Nevertheless, the experimental work follows a logical sequence, beginning with the evaluation of substrate pretreatment by SSF, followed by the BHP test, the modified BHP test, the BMP test, and finally process scale-up in batch and sequential batch reactors. It should be noted that the first two experimental stages were conducted in Monterrey, Mexico, whereas the remaining three were carried out in Narbonne, France.

The materials and methods employed in each experimental stage are described in this chapter.

Additionally, we declare the use of artificial intelligence: ChatGPT (OpenAI, GPT-5.6 Luna) and Grammarly (v.1.2.287.1943) exclusively for language-related purposes, including proofreading, spelling, and grammar correction. These tools were not used to generate scientific results, perform data analysis, interpret experimental results, or develop the scientific content of this thesis. All AI-assisted corrections were reviewed and edited by the author of this work.

3.1. Substrate characterization

In the present work, two different mixtures of lignocellulosic substrates were prepared for the BHP (biohydrogen production potential) and modified BHP tests. The first mixture was composed of fruit peels (FRP), including banana peel (BP, 20%

w/w), melon peel (MP, 20% w/w), and pineapple peel (PP, 10% w/w), combined with brewers' spent grain (BSG, 50% w/w). The second mixture, used for the modified BHP test, consisted of BP (20% w/w), MP (20% w/w), PP (20% w/w), BSG (20% w/w), and wheat straw (WS, 20% w/w).

For the first mixture established above, the FRP were obtained from a restaurant near UANL in San Nicolás de los Garza, Nuevo León, México. On the other hand, BSG was kindly provided by the Heineken plant located in Monterrey, Mexico.

Regarding the second mixture, the fruit peels as well as the WS were obtained from a local supermarket located in Narbonne, France, while the same batch of BSG used in the first mixture was also employed in this experiment.

Once the substrates were obtained, they were dried for 72 h at 80 °C, ground, and sieved to a particle size of 1 mm. Physicochemical parameters such as pH, reducing sugars (RS), total nitrogen (TN), total solids (TS), and volatile solids (VS) were determined for each individual substrate. Following the individual characterization, each mixture was prepared according to the previously established proportions and stored in airtight bags inside desiccators to prevent moisture absorption.

3.2. Substrate Pretreatment Through Solid-State Fermentation (SSF)

Once the lignocellulosic waste mixture was prepared, and with the aim of evaluating the effect of pretreatment on it, pretreatment by solid-state fermentation (SSF) was assessed using the first lignocellulosic waste mixture established in **Section 3.1**. In this experimental stage, three variables were evaluated: temperature (28 °C and 32 °C), time (15 d, 25 d, and 35 d), and fungal strain (*Trametes versicolor* obtained from Fungi Monterrey TV-38 strain and *Pleurotus ostreatus* DSM 1833 from DSMZ), and *Trametes versicolor* (CIRM-BRFM 277, Marseille, France) for replicating the pretreatment assays when the modified BHP test and the 3 L reactors were carried out. Thus, a 3x2x2 mixed factorial design was carried out (**Table 3.1**). Experiments were performed in duplicate, resulting in a total of 24 experiments to evaluate the optimal pretreatment conditions for the lignocellulosic biomass mixture.

Pretreatment effectiveness was assessed through the percentage of delignification and the concentration of reducing sugars, using Klason lignin determination and reducing sugar quantification by the DNS method, respectively, looking for a pretreatment condition that degrades the greatest amount of lignin while preserving the reducing sugar concentration as much as possible. Additionally, total carbohydrates (TCH) concentration was monitored at the different sampling times established using the Dubois method (Dubois et al., 1956). Standard operating conditions during SSF were at an initial pH of 4.2–5.2, dark conditions, and a moisture content of 70%.

Table 3.1. 3x2x3 mixed factorial design employed for the SSF pretreatment stage of the lignocellulosic waste mixture.

Temperature (°C)	Time (d)	Fungus
28	15	*T. versicolor*
28	15	*P. ostreatus*
28	25	*T. versicolor*
28	25	*P. ostreatus*
28	35	*T. versicolor*
28	35	*P. ostreatus*
32	15	*T. versicolor*
32	15	*P. ostreatus*
32	25	*T. versicolor*
32	25	*P. ostreatus*
32	35	*T. versicolor*
32	35	*P. ostreatus*

SSF was carried out in 125 mL Erlenmeyer flasks, using 20 g of the mixture in dry weight adjusted to 70% moisture by the addition of distilled water and subsequently sterilized at 121°C for 15 min at 15 psi before the inoculation with mycelia of each fungus.

To obtain the mycelia of *Trametes versicolor* and *Pleurotus ostreatus*, both fungi were first grown at 28 °C on Petri dishes containing potato dextrose agar (PDA; potato starch 4 $g \cdot L^{-1}$, dextrose 20 $g \cdot L^{-1}$, agar 15 $g \cdot L^{-1}$; BD Bioxon). After complete colonization of the PDA plates, three small agar plugs (~5 × 5 mm) containing

actively growing mycelium were transferred into a liquid medium composed of 2% (w/v) dextrose and 2% (w/v) malt extract prepared with distilled water. The fungal cultures were incubated in the liquid medium for 10 days at 28 °C (**Figure 3.1**). Following incubation, the resulting mycelial suspension was employed as inoculum for SSF by adding a volume corresponding to 10% (v/w) to the previously sterilized substrate (121 °C, 15 min, 15 psi), which was cooled to room temperature beforehand.

Similarly, duplicate control flasks containing only the sterilized substrate at 70% moisture and without mycelial inoculation were prepared. Both control and WRF flasks were incubated under dark and static conditions for the duration and at the temperature of each pretreatment period (**Figure 3.2**).

The effect of each variable included in the 3x2x2 mixed factorial design (time, temperature, and fungal strain), as well as their interactions, on delignification and reducing sugar concentration was evaluated through factorial analysis of variance (ANOVA) using Minitab 18 software. Statistical significance was considered at $p < 0.05$ for all analyses.



Figure 3.1. Suspension of *T. versicolor* and *P. ostreatus* in 2% dextrose and malt extract



Figure 3.2. 125 mL Erlenmeyer Flasks used for SSF

Since the experimental work was conducted in two different locations as previously mentioned, the pretreatment assays performed in Narbonne, France (for the modified BHP tests, the 3 L reactor experiments as well as the BMP test) were

carried out using *Trametes versicolor* CIRM-BRFM 277 (Marseille, France). This strain replaced the Mexican isolate (TV-38 from Fungi MTY) because results in this section identified *T. versicolor* as the most suitable fungal species for biomass pretreatment in the tested conditions.

3.3. BHP test

Once the most suitable pretreatment condition was determined through the analysis of the 3x2x2 mixed factorial design, a BHP test was conducted using both the raw substrate and the substrate pretreated under the optimal conditions identified in the previous stage, following the methodology described below.

3.3.1. Inoculum

For this first BHP test, a microbial consortium derived from granular anaerobic sludge (GAS) collected from a wastewater treatment plant located in Monterrey, Mexico, was employed as inoculum for the dark fermentation process. Subsequently, TS and VS were determined according to standard methods (APHA, 2005). In order to suppress methanogenic activity and promote the enrichment of hydrogen-producing bacteria (HPB), the inoculum underwent a thermal pretreatment at 100°C for 30 min. This pretreatment selectively inhibits methanogenic microorganisms while favoring the survival of spore-forming hydrogen-producing bacteria, as previously described by Cruz-López et al., 2022.

3.3.2. BHP test setup

In this stage of the study, the fungal-pretreated substrate, as well as the control substrate without fungal pretreatment, was sterilized after pretreatment (15 min at 121 °C and 15 psi). Both substrates were then mixed with distilled water (40% w/v), centrifuged at 5000 rpm for 10 min, and subsequently filtered through Whatman No. 1 filter paper (Cat. No. 1001-110) using Kitasato flasks and a Marathon Electric vacuum pump (Model 5KH36KNA510X) to obtain the hydrolysate used as a substrate in the DF assay.

A set of reactors was assembled in 124 mL glass serum bottles containing 80 mL of hydrolysate as working volume. Two reactor groups were evaluated to determine the biochemical hydrogen potential (BHP): one using hydrolysate obtained from the

raw substrate (control) and another using hydrolysate derived from the pretreated substrate selected under the optimal pretreatment condition based on delignification and reducing sugar (RS) concentration. The assays were performed according to the modified methodology proposed by Carrillo-Reyes et al., 2020. employing a substrate-to-inoculum ratio (S/I) of 2.7.

Physicochemical parameters, including RS, pH, TS, and VS, were determined at the beginning and at the end of the process. DF experiments in this BHP test were conducted in duplicate and maintained until H_2 production reached its stationary phase. All reactors were operated under initial conditions of pH 5.50 0.05 using NaOH 2 M and HCl 2 M buffers, 37°C, and 150 rpm. To ensure anaerobic conditions, the headspace was flushed with N_2 gas for 1 minute.

3.3.3. Analytical methods

Throughout the different experimental phases of the project, physicochemical parameters have been monitored *in situ*, including pH, RS, TCH, TS, VS, TN, and Klason lignin.

For individual substrates as well as in the first mixture prepared, SSF, and first BHP assay, characterization of the following parameters was determined: (a) pH, using an Orion model potentiometer, Thermo Scientific brand; (b) TCH using the Dubois method (DuBois et al., 1956); (c) RS employing the DNS (Miller's) methods, a colorimetric method based on the reduction of DNS by glucose or fructose to 3-amino-5-nitrosalicylic acid in an alkaline solution (Miller, 1959). For this method, a calibration curve using glucose as standard was prepared; (d)TN using the standardized Hach method (Hach kit 10-150 $mg \cdot L^{-1}$). Additionally, Klason lignin content for SSF and TS, as well as VS for DF were characterized. Klason lignin was determined following the methodology reported in TAPPI 222 standard (Tappi, 2006), using **Equation 3.1** to calculate the delignification after fungal pretreatment through SSF. Finally, TS and VS for DF were analyzed according to standard methods (APHA, 2005). TCH, RS, and TN measurements were carried out using a UV-vis PerkinElmer Lambda 365 spectrophotometer.

$$Delignification = \left(\frac{(L_0 - L_a)}{L_0}\right) * 100 \qquad \textbf{Equation 3.1}$$

Where L_a is the dry weight lignin fraction (%) of the lignocellulosic mixture after fungal pretreatment, and L_0 is the lignin fraction (%) of the lignocellulosic mixture before fungal pretreatment.

Regarding the biogas composition and volatile fatty acids (VFAs) profiles, they were determined using gas chromatography (GC), using a 7820A, Agilent Technologies GC. The biogas composition was determined using a thermal conductivity detector (TCD), a silica capillary column (30 m x 0.53 mm, Supelco Carboxen 1006 plot), with Argon as the gas carrier. In this case, the gas chromatograph was operated with an oven temperature of 40 °C, while both the injector and detector were maintained at 180 °C. Calibration curves were established using certified gas standard mixtures containing H_2, CH_4, CO, and CO_2 at known concentrations. On the other hand, VFAs were determined using a flame ionization detector (FID) with an Inowax column (50 m x 0.20 mm), with Argon as the gas carrier.

Biogas samples generated during the DF process from the control hydrolysate (C-H) and the fungal-pretreated hydrolysate were monitored one to two times per day until H_2 production ceased (72 h). In contrast, volatile fatty acids (VFAs) were determined only at the end of the fermentation period (72 h). As previously indicated, physicochemical parameters were evaluated at both the beginning and the end of the DF process.

Once the fermentation stopped, **Equation 3.2** was used to determine the hydrogen yield (YH_2), which was calculated as the volume of H_2 produced (VH_2) in mL, divided by the initial content of VS (VS_i) in g.

$$YH_2 = \frac{VH_2}{VSi} \qquad \textbf{Equation 3.2}$$

In order to describe the kinetics of H_2 production throughout the BHP test, hydrogen production data obtained in this BHP test were processed using a modified Gompertz function (**equation 3.3**)(Reyna-Gómez et al., 2019):

$$H(t) = Hmax * \exp[-exp\left(\frac{2.71828 * Rmax(\lambda - t)}{Hmax} + 1\right)] \quad \textbf{Equation 3.3}$$

where H(t) represents the cumulative hydrogen production (mL) at a specific time t (h); Mmax (mL) corresponds to the maximum hydrogen production achieved; Rmax (mL h^{-1}) denotes the maximum hydrogen production rate; and λ (h) indicates the lag-phase duration prior to the exponential phase of hydrogen production. Data analysis was performed in Excel using the Solver function.

Additionally, hydrogen production efficiency (HPE) was determined using **Equation 3.4**. This parameter compares experimental H_2 production with theoretical H_2 production based on acetic and butyric acid formation on a molar basis. This analysis estimates whether acetic acid is produced through the homoacetogenic pathway or whether it is associated with hydrogen production (Perat et al., 2024).

$$HPE = \frac{H_2 experiment}{2(Acetic\ acid + butyric\ acid)} \quad \textbf{Equation 3.4}$$

Where H_2 experiment (mol·L^{-1}) is the cumulative hydrogen produced at the end of the process, and acetic/butyric acid refers to the cumulative amount of each VFA produced at the end of the process (mol·L^{-1}).

3.3.4. DNA extraction and microbial identification

The microbial communities in each group of this BHP test (control and fungal-pretreated substrate-fed systems) were determined. For this analysis, samples from each experimental group were collected and subjected to microbial community characterization. Genomic DNA was extracted from 0.25 g of sample using the QIAGEN DNeasy PowerSoil Kit, following the manufacturer's protocol (Cruz-López et al., 2022). Cell disruption was achieved through combined mechanical and chemical lysis, enabling the release of intracellular genetic material. Nucleic acids were subsequently adsorbed using salt-based reagents, and genomic DNA was

retained on a silica membrane, followed by washing and elution steps after centrifugation (30 s at 10,000xg)(Casanova-Mina et al., 2023).

To determine the microbial composition of the dark fermentation systems, amplicon sequencing coupled with PCR-based sequencing was performed by RTL Genomics (Texas, USA). Microbial profiling was carried out through 16S rRNA gene sequencing using an Illumina MiSeq platform with the primers 28F (GAGTTTGATCNTGGCTCAG) and 519R (GTNTTACNGCGGCKGCTG), generating approximately 10k reads per sample (Cruz-López et al., 2022).

Sequence processing included the removal of chimeric, singleton, short, and low-quality sequences, as well as sequencing noise, using the USEARCH algorithm and UCHIME software. Sequences longer than 250 bp and with a minimum quality score of 30 were retained for further analysis. Operational taxonomic units (OTUs) were defined at 97% sequence identity using the UPARSE algorithm. Finally, taxonomic assignment of each OTU was performed through alignment against a curated high-quality database derived from NCBI and maintained internally by RTL Genomics.

3.4. Modified BHP test

After analyzing the results obtained from the BHP test and with the aim of improving them, a modified BHP test was subsequently carried out by changing some operational parameters, beginning with the composition of the lignocellulosic mixture used as substrate. In this case, the second lignocellulosic waste mixture established in **Section 3.1** was employed, together with the process adjustments described below

3.4.1. Inoculum

The inoculum was changed in this modified BHP test, since aerobic sludge (AS) was used in this experimental stage. AS was collected from the wastewater treatment plant in Narbonne, France. Samples were collected in plastic containers and subsequently centrifuged at 10,000 rpm for 10 min. The supernatant was discarded, and the pellet was used as inoculum. TS and VS were determined according to standard methods (APHA, 2005). As in the GAS used in the previous

BHP test, the AS was subjected to a thermal treatment at 100°C for 30 min to suppress methanogen archaea proliferation.

3.4.2. Modified BHP test setup

The substrate used for the modified BHP test consisted of the second lignocellulosic mixture, prepared and conditioned under the same conditions as the first one (see **Section 3.1**). The conditions of fungal pretreatment through SSF were the same as in the first BHP test, carrying out the most suitable pretreatment condition observed in **Section 3.2**.

In this experimental stage, the modified BHP test was carried out in duplicates. For this purpose, the fermentation was done in batch mode using 600 mL bottles with a working volume of 200 mL at 37°C using an agitation of 150 rpm. All reactors were set up at an initial concentration of <10 $gVS \cdot L^{-1}$ and a S/I 20. Both systems, raw and fungal-pretreated, were added directly to the fermentation bottle, adjusting the working volume by adding distilled water. The pH was established at 5.50 ± 0.05 using NaOH 2 M and HCl 2 M buffers. To ensure anaerobic conditions, the headspace was flushed with N_2 gas for 1 minute, and subsequently the bottles were sealed with butyl rubber stoppers and aluminum caps.

3.4.3. Analytical methods

To determine the physicochemical characteristics of each substrate used in the second lignocellulosic mixture, as well as those of each group from the modified BHP test and the subsequent scaled-up reactors, a series of parameters were analyzed. pH was measured using a Mettler Toledo potentiometer (FiveEasy model). TN for the lignocellulosic mixture used in the modified BHP test, batch reactors, and sequencing batch reactors was determined using the standardized Hach method with the LCK338 kit (20–100 $mg \cdot L^{-1}$). RS were quantified by the DNS method described in **Section 3.3.3**, as in the same manner for TS and VS, which were determined according to standard methods (APHA, 2005). Hydrogen yield and HPE were calculated using **Equations 3.2** and **3.4**, respectively

In this modified BHP test, the biogas composition (H_2, CO_2, O_2, and CH_4) was analyzed every two hours during dark fermentation. For this purpose, a micro gas

chromatograph (μGC, SRA 1-GC R3000) was used, equipped with a 10 m Molsieve (PPU) column operated at 80 °C using argon as the carrier gas, and an 8 m VAR (PPU) column maintained at 70 °C with helium as the carrier gas for the analysis of O_2, CH_4, H_2, and N_2, and CO_2, respectively. The thermal conductivity detector (TCD) was maintained at 90 °C. as previously described in (Ben Yahmed et al., 2021). The fermentation stopped once the biogas production reached the stationary phase.

Regarding the VFAs, they were analyzed using a PerkinElmer gas chromatograph (Clarus 580) equipped with an Alltech FFAP ECTM 1000 column and a flame ionization detector (FID), using N_2 as the carrier gas (flow of 6 $mL \cdot min^{-1}$) (Noguer, Escudié, et al., 2022). All samples were collected at the end of the fermentation and filtered through a 0.2 μm nylon membrane filter before analysis.

3.4.4. DNA Extraction and Microbial Identification

For the modified BHP test, scaled-up batch reactors, and sequencing batch reactors, the microbial identification was done by taking 2 mL of liquid sample at the end of the process/cycle. Samples were centrifuged at 13,000 rpm for 15 min, after which the supernatant was discarded, and the resulting pellet was transferred into sterile 2 mL Eppendorf tubes. Pellets were preserved at -20 °C until DNA extraction.

Genomic DNA extraction was performed using the FastDNA SPIN Kit for Soil according to the manufacturer's recommendations (MP Biomedicals, California, USA). DNA concentration and purity were evaluated by spectrophotometry using an TecanInfinite NanoQuant M200 instrument, and purified DNA samples were subsequently stored at -20 °C.

Amplification of the V3–V4 hypervariable region of the 16S rRNA gene was carried out by polymerase chain reaction (PCR) employing universal primers previously reported in (Carmona-Martínez et al., 2015). The PCR mixture, with a final volume of 60 μL, consisted of iProof™ High-Fidelity DNA Polymerase (Bio-Rad), reaction buffer, forward primer 344F (5'-ACGGRAGGCAGCAG-3'), reverse primer 802R (5'-TACCAGGGTATCTAATCCT-3'), dNTPs, template DNA, and nuclease-free water.

PCR amplification was conducted in a Mastercycler thermal cycler (Eppendorf, Germany) using an initial denaturation step at 98 °C for 1 min, followed by 30 amplification cycles consisting of denaturation at 98 °C, annealing at 65 °C, and extension at 72 °C, each for 1 min. A final extension step was performed at 72 °C for 10 min.

Amplification success and amplicon quality were verified using the Agilent 2100 Bioanalyzer. Sequencing analyses were conducted on an Illumina MiSeq platform generating 2 x 300 bp paired-end reads at the GenoToul genomics facility (Toulouse, France).

Raw sequencing reads were processed using bioinformatics pipelines including quality filtering, denoising, paired-end assembly, and chimera removal with mothur v1.48.0. Sequence alignment and taxonomic assignment were performed against the SILVA database (release 132).

3.5. BMP test for biodegradability determination

Following the evaluation of the hydrogen production potential in the modified BHP test using the second lignocellulosic mixture described in **Section 3.1**, the biochemical methane potential (BMP) test was performed to determine the biodegradability of the lignocellulosic mixture used as substrate, both with and without fungal pretreatment.

Before the BMP test, the chemical oxygen demand (COD) of both substrates (raw and fungal-pretreated) was determined using the Lovibond COD Vario tube test tubes (0-1500 $mg \cdot L^{-1}$) and a uv-vis spectrophotometer Aqualytic MultiDirect with a wavelength of 600 nm. For this purpose, substrate suspensions were prepared at concentrations of 1% (w/v) for the raw substrate (RAW MIX) and 3% (w/v) for the fungal-pretreated substrate (SSF25d MIX). Additionally, the VS content of each suspension was determined according to Standard Methods (APHA, 2005) in order to establish the COD/VS ratio of each substrate.

Subsequently, three groups of reactors were set up in triplicate: blank, raw substrate, and substrate pretreated with *T. versicolor* for 25 d at 28 °C. Granular

anaerobic sludge (GAS) was used as inoculum without any pretreatment. The BMP assays were conducted in 600 mL serum bottles with a working volume of 400 mL. All reactor groups were operated under the same conditions: 35 °C, 150 rpm, and 40 d of incubation. The blank reactors contained only inoculum (200 mL) and 200 mL of distilled water supplemented with 2.5 $g \cdot L^{-1}$ of $NaHCO_3$. This reactor group was included to determine the endogenous methane production of the inoculum.

The reactor groups containing raw and fungal-pretreated substrates were operated at an S/I 0.5, using 1 gVS of substrate and 2 gVS of inoculum. These reactors were maintained under the same operating conditions of temperature, agitation, working volume, and buffer concentration (35 °C, 150 rpm, 400 mL, and 2.5 $g \cdot L^{-1}$ $NaHCO_3$). For both substrate-containing groups, the working volume was completed with distilled water. After loading the bottles with inoculum, substrate, and distilled water, the headspace was purged with N_2 gas for 1 min to ensure anaerobic conditions.

Biogas production and composition were monitored during all BMP test for 40 days, using a pressure gauge and a gas chromatograph, respectively. The gas chromatograph used in this study was a PerkinElmer Clarus 480 gas chromatograph equipped with a thermal conductivity detector (TCD). Helium (He) was used as the carrier gas, and separation was performed using an Rt-U-BOND PLOT column (30 m x 0.32 mm). The system could detect CO_2, H_2S, O_2, N_2, and CH_4.

After 40 d of incubation, the biodegradability of both substrates was determined using **Equations 3.5**, **3.6**, **3.7**, and **3.8**, which are described below.

$$COD_{added}\ (\mathrm{g}) = VS\ added\ .\ (\frac{COD}{VS}) \qquad \textbf{Equation 3.5}$$

Where VS added refers to the total amount of initial volatile solids (VS) introduced into the system, considering both the inoculum and substrate contributions (3 g), and COD/VS represents the amount of chemical oxygen demand (COD) per gram of volatile solids ($gCOD \cdot gVS^{-1}$), previously determined in the prepared solutions of substrate mixtures (raw and fungal pretreated).

$$Vol\ CH_4\ net\ (L) = vol\ CH_4 - \ vol\ CH_4\ blank \qquad \textbf{Equation 3.6}$$

Where Vol CH_4 (L) refers to the volume of methane produced by the substrate under evaluation, and Vol CH_4 blank corresponds to the volume of methane generated endogenously by the inoculum, as determined from the blank reactors during the BMP assay (L).

$$Biodegradable\ COD\ (g) = \frac{vol\ CH_{4\ net}}{0.39} \qquad \textbf{Equation 3.7}$$

Where Vol $CH_{4\ net}$ refers to the cumulative methane volume obtained from the experimental reactors after correction for the endogenous methane production observed in the blank reactors (expressed L). The factor 0.39 (L $CH_4 \cdot gCOD^{-1}$) corresponds to the theoretical methane yield associated with the complete conversion of 1 g of COD into methane at 35 °C and 1 atm. This factor was calculated from the ideal gas law and the stoichiometric relationship whereby 1 mol of CH_4 is equivalent to 64 g of COD.

$$Biodegradability\ (\%) = \left(\frac{Biodegradable\ COD}{COD_{added}}\right) . 100 \qquad \textbf{Equation 3.8}$$

Where biodegradable COD corresponds to the value obtained from **Equation 3.7** (expressed in g), and COD added corresponds to the value calculated using **Equation 3.5** (expressed in g).

3.6. Process Scaling up in Batch and Sequencing Batch Reactors

Following the evaluation of both BHP tests, process scale-up in batch reactors was carried out using the fungal pretreatment condition that exhibited the best performance in terms of delignification and RS concentration.

3.6.1. Inoculum

The inoculum used in this experimental stage consisted of thermally pretreated AS, as described in **Section 3.4.1,** using an S/I 20 based on the VS concentration of both the substrate and inoculum.

3.6.2. Experimental Setup of the Batch Reactors

Two different batch reactors were assembled using 3 L vessels with a working volume of 2 L. The first system consisted of a bioreactor fed with raw, non-pretreated substrate (raw batch reactor, RBR), while the second bioreactor contained substrate pretreated by SSF (fungal-pretreated batch reactor, FPBR). The characteristics of each assembled batch bioreactor are presented in **Figure 3.3**.



RBR: Raw substrate (lignocellulosic mixture, pH 5.50 ± 0.05, VS < 10 $g \cdot L^{-1}$, 150 rpm, 37°C, 48 h)



FPBR: Fungal-pretreated substrate (lignocellulosic mixture, pH 5.50 ± 0.05, VS < 10 $g \cdot L^{-1}$, 150 rpm, 37°C, 48 h)

Figure 3.3. Operational conditions of batch reactors.

The purpose of the RBR and FPBR systems was to compare the impact of fungal pretreatment on hydrogen and VFA production under scaled-up conditions with controlled pH, agitation, and hydrogen partial pressure, aiming to improve the performance observed in the two previously conducted BHP tests. Both reactors were operated under the same conditions: VS < 10 $g \cdot L^{-1}$, controlled pH of 5.50 ± 0.05, agitation at 150 rpm, temperature of 37 °C, and reaction time of 48 h. Each reactor was equipped with pH and oxygen sensors. To ensure anaerobic conditions, the headspace was flushed at the beginning with N_2 gas for 1 minute.

3.6.3. Experimental Setup of the Sequencing Batch Reactors

After operating the 3 L batch reactors and analyzing their results, two sequencing batch reactors were subsequently assembled with the objective of not only

evaluating the system performance in terms of hydrogen and VFAs production but also assessing its robustness and determining how many feeding cycles the system could withstand without additional inoculum supplementation.

For this purpose, two systems were once again established. The first system contained raw substrate (raw sequencing batch reactor, RSBR), whereas the second system used the lignocellulosic mixture pretreated with fungi through SSF as substrate (fungal-pretreated sequencing batch reactor, FPSBR). Both systems were operated in the same 3 L reactors, now using a working volume of 1.5 L.

Each system was initially filled following the same procedure used for the batch reactors operated in the previous experimental stage and allowed to undergo DF for 48 h. Subsequently, two-thirds of the reactor volume was withdrawn and replaced with fresh substrate using a peristaltic pump connected to the reactor. At the beginning of the process as well as each feeding cycle, the headspace was flushed with N_2 gas for 1 minute to ensure anaerobic conditions. The feeding cycles were carried out until hydrogen production in the system reached the stationary phase. A graphical representation of this process is presented in **Figure 3.4**.

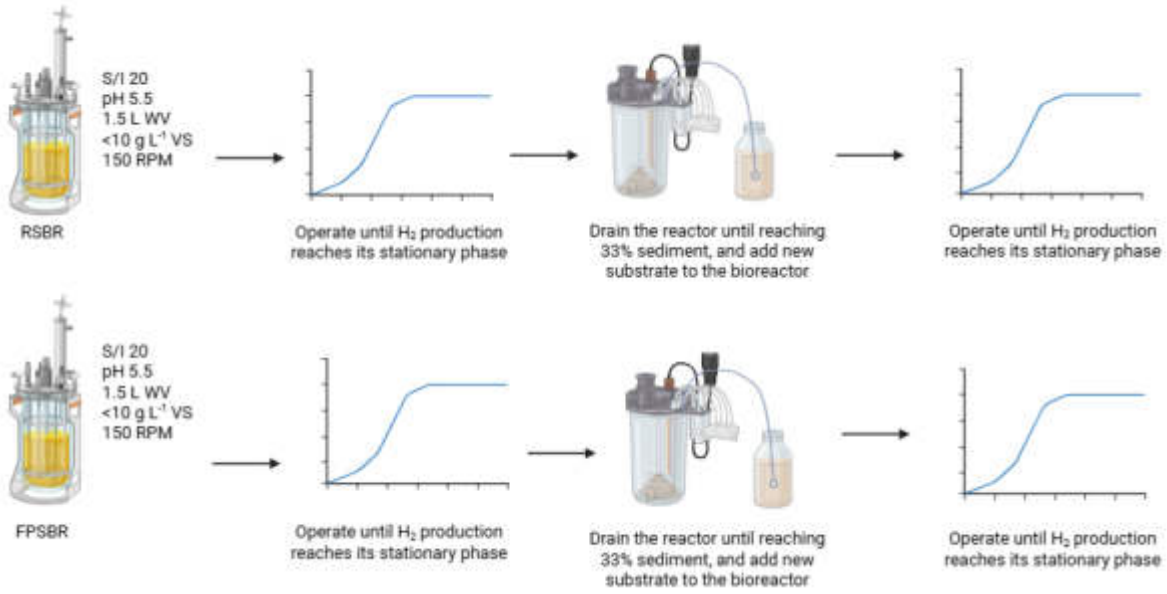


Figure 3.4. Schematic representation of DF process in sequencing batch reactors. (37°C, pH 5.50 ± 0.50, 150 rpm, VS < 10 g·L^{-1}, anaerobic sludge as inoculum, S/I 20).

3.6.4. Analytical methods

The analytical methods employed in this experimental stage were the same as those used in the modified BHP test (**Section 3.4.3**). pH, RS, TS, and VS were measured at the beginning and at the end of the fermentation process or feeding cycle.

Biogas composition was analyzed once the internal pressure of the bioreactor reached 1,070 mbar, using the same system described in **Section 3.4.3**.

3.6.5. DNA Extraction and Microbial Identification

DNA extraction and the subsequent identification of microbial communities in the batch and sequencing batch reactors were carried out following the same methodology described in **Section 3.4.4**.

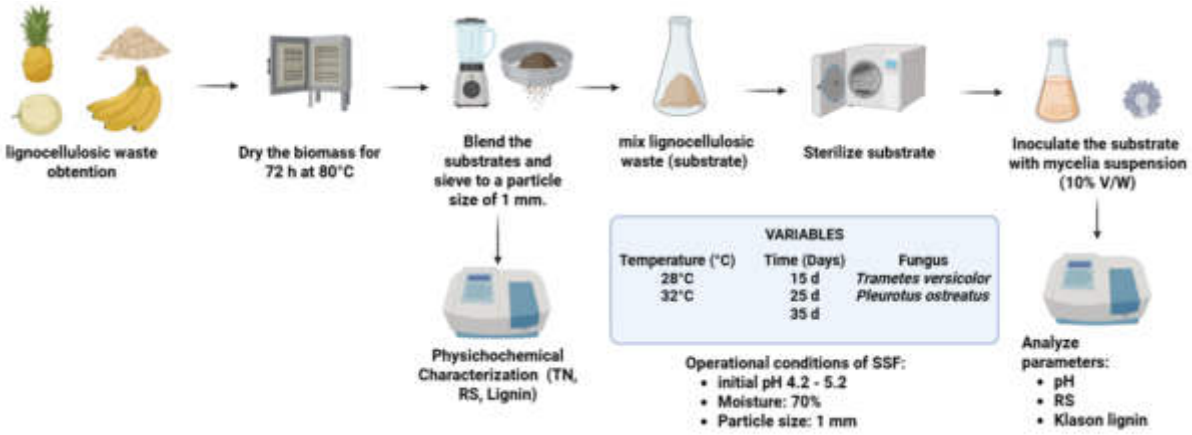
3.7. Experimental diagram

Figure 3.5 (a) presents a general schematic representation of the experimental methodology followed in this study, which involved two main bioprocesses: SSF, used for the pretreatment of the previously established lignocellulosic substrate mixture, and DF, aimed at hydrogen and VFA production. **Figure 3.5 (b)** presents a block diagram providing an overall overview of all the experimental stages carried out throughout this research.

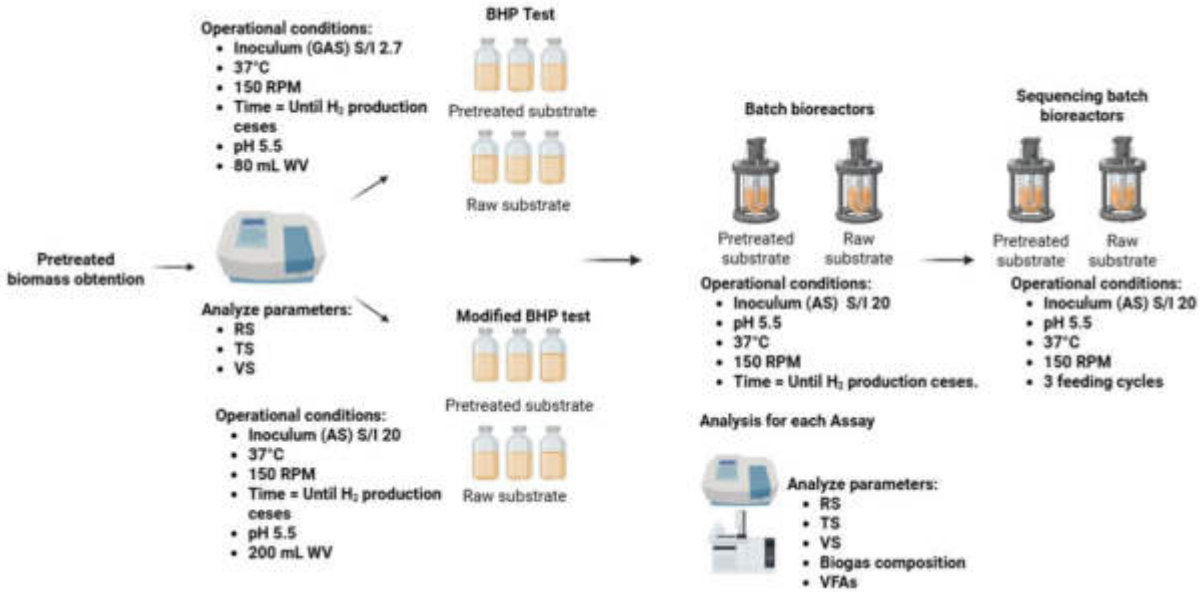
The first experimental stage focused on evaluating different pretreatment conditions and their effects on Klason lignin content and reducing sugar concentration. The second stage involved the BHP assays, followed by process scale-up under two different operational modes: batch and sequencing batch.

a

Solid-State Fermentation



Dark Fermentation



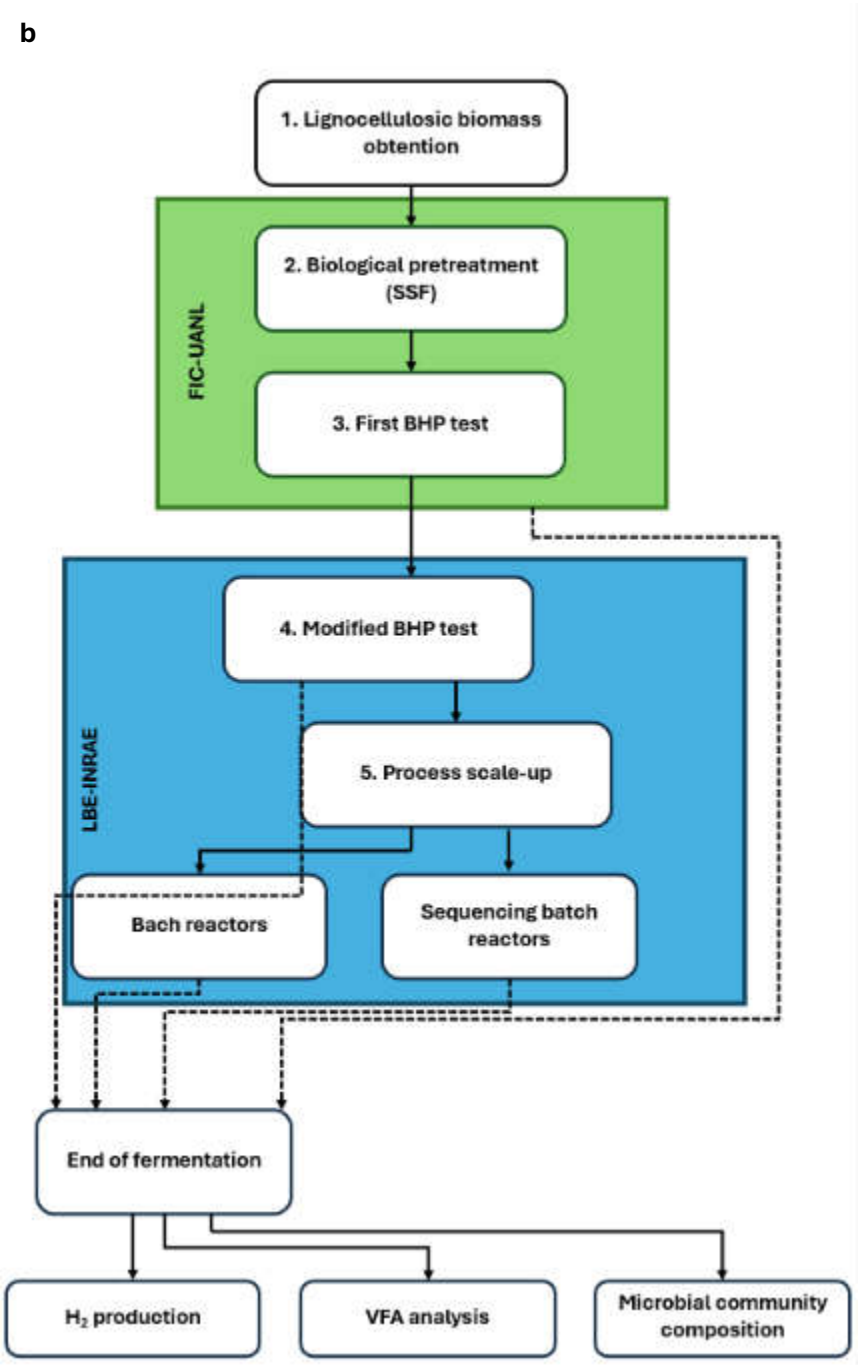


Figure 3.5. (a) General diagram of both bioprocesses carried out: Biological pretreatment through SSF and H_2 production through DF. (b) Block diagram illustrating the experimental workflow followed throughout this research.

CHAPTER 4

4. Results in solid-state fermentation, experimental design analysis, and BHP test

The results presented in this section have been published in the journal Biomass and Bioenergy (volume 210): https://doi.org/10.1016/j.biombioe.2026.109040

4.1. Substrates characterization

Tables 4.1 and **4.2** show the physicochemical characteristics (TN, RS, pH, TS, and VS) of the substrates used in the BHP test and the modified BHP test, respectively. The concentrations of each individual substrate (BP, MP, PP, and BSG), as well as those obtained after mixing them according to the proportions previously established in **Section 3.1** of Materials and Methods (MIX 1), are presented.

As can be observed in the first mixture used for the BHP test, each substrate individually contributed different concentrations of RS, varying among substrates, with BP presenting the highest RS concentration and BSG the lowest. After mixing all lignocellulosic wastes in MIX 1 according to the previously established ratio, an RS concentration of 25.49 ± 5.53 $g \cdot Kg^{-1}$ was obtained. Regarding TN, the mixture showed a concentration of 11.29 ± 0.20 $g \cdot Kg^{-1}$, and a pH of 4.89 ± 0.05, which favors fungal growth during SSF without the need for buffer addition, since WRF are able to grow optimally within a pH range of 3.5 - 5.5 (Kainthola et al., 2021). With respect to TS and VS, all substrates showed similar values, ranging from 94.5% to 99.0% of TS and 88.10% to 95.43% of VS.

Table 4.1. Physicochemical characterization of individual substrates and the first mixture (MIX 1) before fungal pretreatment (20% BP, 20% MP, 10% PP, and 50% BSG).

Substrate	TN (g·Kg-1)	RS ($g \cdot Kg^{-1}$)	pH	TS ($g \cdot Kg^{-1}$)	VS ($g \cdot Kg^{-1}$)
BP	17.73 ± 0.53	199.23 ± 1.06	5.30 ± 0.01	969.05 ± 0.56	881.67 ± 1.31
MP	15.90 ± 0.28	76.54 ± 1.71	4.59 ± 0.03	956.83 ± 0.09	953.83 ± 0.09
PP	6.93 ± 0.18	134.78 ± 4.14	3.85 ± 0.01	945.11 ± 1.21	903.64 ± 0.58
BSG	15.27 ± 0.07	39.67 ± 13.84	4.82 ± 0.03	990.62 ± 1.14	954.28 ± 0.34
MIX 1	11.29 ± 0.20	25.49 ± 5.53	4.89 ± 0.05	970.12 ± 0.42	894.09 ± 0.70

Regarding the second mixture (MIX 2) used for the modified BHP test, as can be observed in Table 4., all fruit peels as well as WS and BSG, showed TS and VS values similar to those obtained for MIX 1. In terms of RS, WS and BSG were the individual substrates with the lowest concentrations, presenting values of 46.43 ± 8.08 $g \cdot Kg^{-1}$ and 19.29± 6.10 $g \cdot Kg^{-1}$, respectively.

TN values differed from those observed in the first lignocellulosic mixture due to the use of a different methodology for the determination of this parameter (see Sections 3.3.3 and 3.4.3). Nevertheless, all individual substrates, with exception of MP, showed similar TN values. In this case, MP presented the highest TN concentration, reaching 22 ± 0.02 $g \cdot Kg^{-1}$.

Similar to MIX 1, MIX 2 exhibited physicochemical characteristics favorable for fungal growth during the pretreatment stage, with a pH of 5.32 ± 0.06. In addition, MIX 2 showed a higher RS concentration than MIX 1, reaching 197.15 ± 11.11 g·Kg-1. This increase was mainly attributed to the change in substrate proportions during the preparation of the lignocellulosic mixture, since the considerable reduction of the substrate with the lowest RS content (BSG) positively impacted this parameter (Šnajdr & Baldrian, 2007).

Table 4.2. Physicochemical characterization of individual substrates and the second mixture (MIX 2) before fungal pretreatment (20% BP, 20% MP, 20 % PP, 20% WS, and 20% BSG).

Substrate	TN ($g \cdot Kg^{-1}$)	RS ($g \cdot Kg^{-1}$)	pH	TS ($g \cdot Kg^{-1}$)	VS ($g \cdot Kg^{-1}$)
BP	2.4 ± 0.01	250.72 ± 52.53	5.66 ± 0	951.95 ± 5.25	811.84 ± 7.80
MP	22 ± 0.02	176.43 ± 16.16	4.95 ± 0.01	956.83 ± 0.09	953.83 ± 0.09
PP	4.3 ± 0.01	191.43 ± 5.05	4.77 ± 0.01	947.14 ± 2.17	894.86 ± 2.65
WS	3.78 ± 0	46.43 ± 8.08	5.72 ± 0.07	986.92 ± 0.47	906.41 ± 1.79
BSG	3.33 ± 0.01	19.29 ± 6.10	6.22 ± 0.03	990.62 ± 1.14	954.28 ± 0.34
MIX 2	8.43 ± 0	197.15 ± 11.11	5.32 ± 0.06	972.37 ± 0.70	897.70 ± 0.44

4.2. Substrate Pretreatment Through SSF

As mentioned above, MIX 1 was subjected to different pretreatment conditions through SSF with the aim of identifying the most efficient pretreatment in terms of delignification and RS preservation. Additionally, during the SSF, TCH was measured

at the end of each sampling times (15 d, 25 d, 35 d) to gain a better understanding of the process and assess changes in the total sugar content of the mixture throughout the pretreatment.

Figures 4.1 and **4.2** display the TCH and RS concentrations, respectively. Even though duplicate assays limit the statistical power to detect significant interactions between factors, the observed trends were consistent and reproducible, providing reliable insights into the effects of each evaluated pretreatment condition. A constant consumption of TCH and RS throughout pretreatment was evident in all cases, indicating the utilization of not only lignin, but also cellulose and hemicellulose present in the lignocellulosic mixture used as substrate.

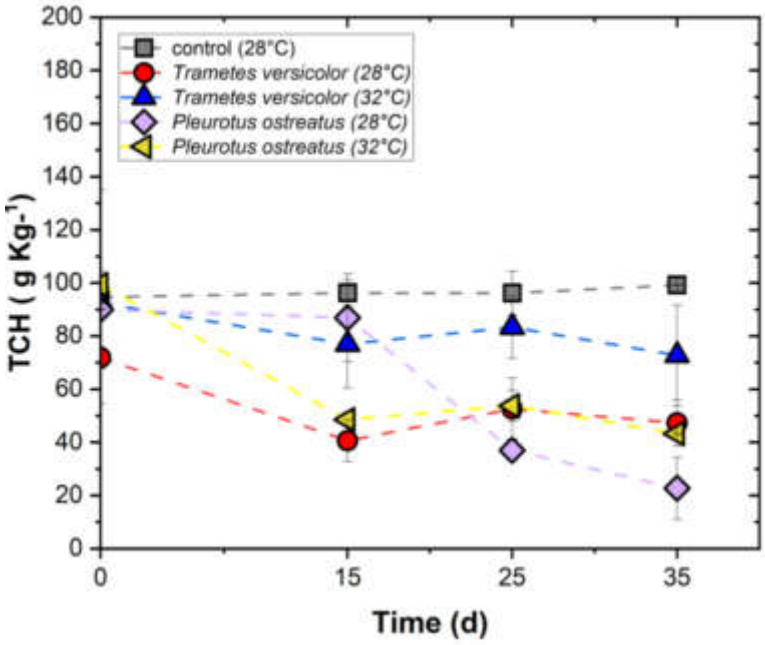


Figure 4.1. Evolution of TCH concentration during the SSF under all pretreatment conditions tested: Fungal strain (*T. versicolor* TV-38 and *P. ostreatus* DSM 1833), temperature (28 °C and 32 °C), and time (15, 25, and 35 d).

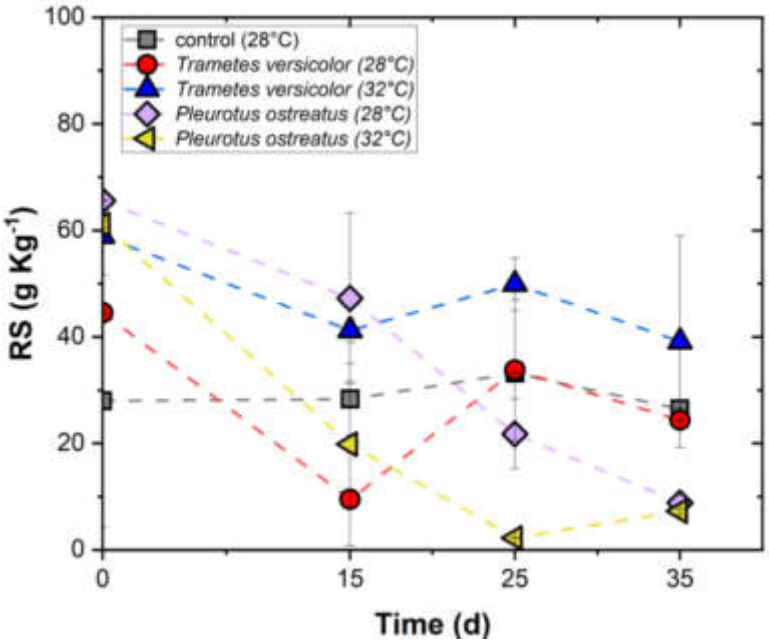


Figure 4.2. Evolution of RS concentration during the SSF under all pretreatment conditions tested: Fungal strain (*T. versicolor* TV-38 and *P. ostreatus* DSM 1833), temperature (28 °C and 32 °C), and time (15, 25, and 35 d).

In the case of *T. versicolor* cultivated at 28 °C, an increase in RS concentration from 9.48 to 33.75 g·kg^{-1} was observed between days 15 and 25. This behavior suggests that *T. versicolor* initially metabolizes the total carbohydrates present in the substrate while simultaneously releasing reducing sugars as a result of the partial hydrolysis of lignocellulosic compounds (Garduño et al., 2024). However, after 35 days of pretreatment, the final concentrations of both TCH and RS were lower than their initial values at both evaluated temperatures (28 °C and 32 °C). Final TCH concentrations of 47.29 ± 8.58 and 72.7 ± 19 g·kg^{-1} were obtained at 28 °C and 32 °C, respectively, whereas RS concentrations reached 24.35 ± 0.21 and 39.11 ± 3.25 g·kg^{-1}, respectively.

In contrast, the pretreatment assays performed with *P. ostreatus* exhibited higher reductions in both TCH and RS under all evaluated conditions compared to those obtained with *T. versicolor*. At 28 °C, the final concentrations of TCH and RS reached 22.67 ± 11.75 and 8.81 ± 1.34 g kg^{-1}, respectively. Similarly, in the 32 °C pretreatment group, final TCH and RS concentrations of 43.23 ± 0.47 and 7.27 ± 0.7 g·kg^{-1},

respectively, were obtained. These results indicate that *Pleurotus ostreatus* behaves as a non-selective lignin degrader, since its enzymatic system is also capable of degrading cellulose and hemicellulose, consequently promoting higher sugar losses in the substrate (Nadir et al., 2019), as presented in **Table 4.3**.

Table 4.3. TCH and RS consumption (%) at each sampling time during SSF using *T. versicolor* TV-38 and *P. ostreatus* DSM 1833.

Strain	Temperature (°C)	Time (d)	TCH consumption with respect to initial concentration (%)	RS consumption with respect to initial concentration (%)
Trametes versicolor	28	15	43 ± 14	79 ± 20
		25	28 ± 12	24 ± 0
		35	31 ± 18	45 ± 0
	32	15	17 ± 0	30 ± 4
		25	10 ± 0	15 ± 8
		35	21 ± 0	33 ± 0
Pleurotus ostreatus	28	15	3 ± 0	28 ± 0
		25	59 ± 4	67 ± 10
		35	67 ± 13	87 ± 2
	32	15	51 ± 0	68 ± 19
		25	46 ± 8	96 ± 0
		35	57 ± 2	88 ± 1

Based on the results presented in the previous table, it can be inferred that the pretreatment carried out using *P. ostreatus* was not consistent with the objective of the study, since considerable reductions in both TCH and RS concentrations were observed in comparison with the groups pretreated using *T. versicolor*.

The above should be related to delignification, as the main objective in this stage is to degrade lignin while preserving the RS for subsequent DF. To address this, **Figure 4.3** shows the delignification under all pretreatment conditions evaluated.

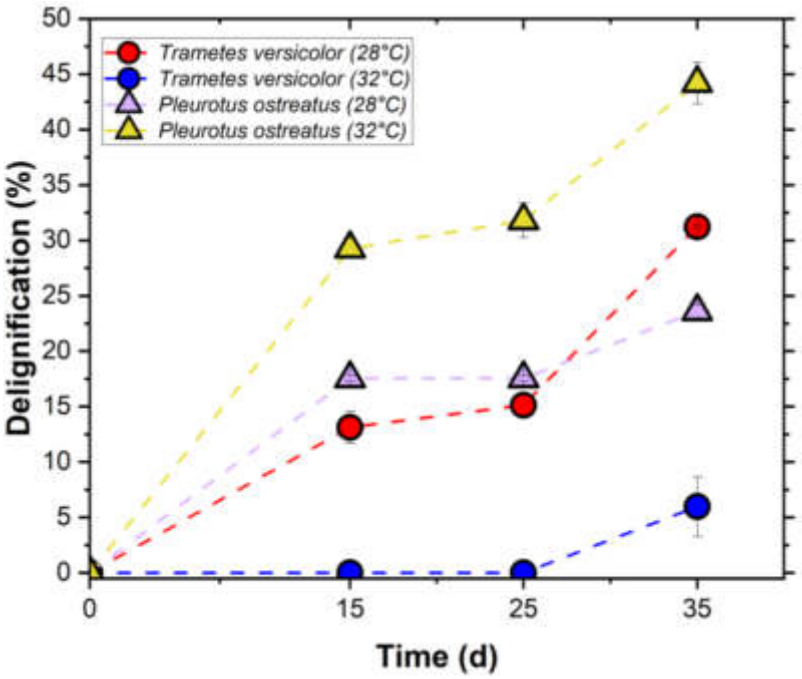


Figure 4.3. Klason lignin degradation in the lignocellulosic mixture through the SSF process using *T. versicolor* TV-38 and *P. ostreatus* DSM 1833. Temperature (28 °C and 32 °C), and time (15, 25, and 35 d).

In the pretreatment assays carried out using *T. versicolor* at 32 °C, important degradation of reducing sugars was observed; however, no considerable delignification was achieved, reaching only 6 ± 2.7 % lignin removal after 35 days of pretreatment. Conversely, the group treated with *T. versicolor* at 28 °C exhibited delignification values of up to 31 ± 0.14 % by the end of the SSF process. This behavior is likely associated with laccase activity, one of the principal enzymes involved in lignin degradation by *T. versicolor*, whose optimal activity has been reported around 28 °C (Nadir et al., 2019). Furthermore, the optimal growth temperature of *T. versicolor* is below 30 °C, which may have directly influenced lignin degradation efficiency, as well as the excessive consumption of TCH and RS observed under these conditions (A. E. Adekunle et al., 2017).

In contrast, the *P. ostreatus* pretreatment groups achieved delignification values of 23.6 ± 0.58 % and 44.2 ± 1.89 % at 28 °C and 32 °C, respectively, by the end of the SSF process. Higher RS consumption and Klason lignin degradation were observed with this strain at 32 °C compared to *T. versicolor*, suggesting that *P.*

ostreatus degrades not only the lignin fraction of lignocellulosic biomass, but also the sugars present in the holocellulose. Although some studies have *described P. ostreatus* as a selective lignin degrader, the results obtained in the present work indicate that the effectiveness and selectivity of the fungal pretreatment strongly depend on the nature and composition of the substrate being treated (Dong et al., 2013; Salame et al., 2014; Suryadi et al., 2022). It is important to consider that, although the Klason lignin method is widely employed for lignin determination, it presents certain limitations associated with its gravimetric basis. Furthermore, residual fungal mycelium remaining in the substrate after pretreatment may contribute to the measured values, potentially influencing the absolute lignin content, especially under conditions where fungal growth differed among treatments (Rese et al., 2025). However, all samples were subjected to identical conditions. Therefore, the differences observed in the lignin fraction mainly reflect the relative trends in lignin degradation induced by the fungal pretreatment rather than exact lignin concentrations.

The delignification values obtained for both fungal strains in the present study were higher than those previously reported by Machado & Ferraz, 2017 who achieved 11.1 % lignin degradation in sugarcane bagasse after 60 days of pretreatment with *P. ostreatus*. Similarly, (Bari et al., 2016) reported lignin degradations of 17.54 % and 20.06 % after 20 days of pretreatment with *P. ostreatus* and *T. versicolor*, respectively, during the degradation of beech wood (*Fagus orientalis*). Nevertheless, as previously discussed, lignin degradation efficiency is strongly influenced by the characteristics and composition of the substrate used.

4.3. Experimental Design Analysis

Considering the results presented in the previous subsection, a factorial analysis of variance (ANOVA) was carried out to determine the most important factors involved in the pretreatment process, using reducing sugar loss (%) and delignification (%) as response variables.

Variance analysis allowed the construction of the Pareto charts shown in **Figure 4.4a** and **b**, considering delignification and reducing sugar loss as response variables, respectively. For delignification, the fungal strain and its interaction with temperature were identified as the factors with a significant effect on this response. In contrast, reducing sugar loss was significantly influenced not only by the fungal strain and the interaction between temperature and strain, but also by the interaction between treatment time and fungal strain during the pretreatment stage.

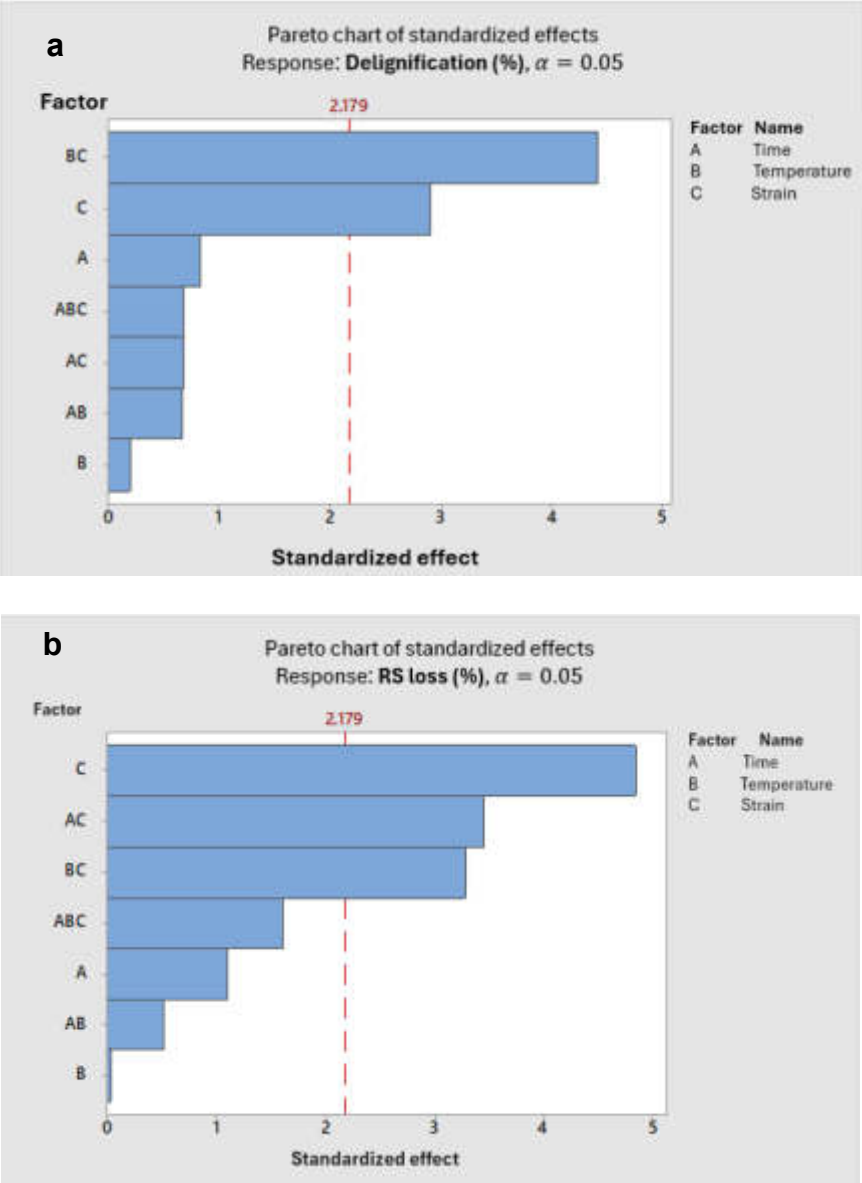


Figure 4.4. (a) Pareto chart of standardized effects to delignification, and (b) Pareto chart of standardized effects to RS loss.

Taking these considerations into account, the optimal conditions were identified to enhance lignin removal while reducing sugar losses to the minimum possible level. Among all the pretreatment conditions evaluated, the best performance was achieved when SSF was carried out with *T. versicolor* at 28 °C for 25 days. Therefore, the substrate pretreated under these conditions was selected for the subsequent dark fermentation process and prepared according to the methodology described in **Section 3.3.2** for the BHP test setup.

4.4. BHP test

After carrying out the pretreatment for 25 d using *T. versicolor* TV-38 at 28°C, the prepared hydrolysate was used as substrate for H_2 production (TV-H). **Table 4.4** displays the initial physicochemical characteristics of the two groups of reactors set up in this experimental stage, including the control (hydrolysate without fungal pretreatment, C-H) and the *T. versicolor*-pretreated hydrolysate (TV-H). The main objective in the BHP test was to evaluate the impact of the pretreatment on H_2 yield as well as on the VFA production.

Table 4.4. Initial physicochemical characteristics determined for each group of reactors used in the BHP test: Control (C-H), and *T. versicolor* hydrolysate for 25 d at 28 °C (TV-H).

Reactor	Inoculation ratio (S/I)	TS ($g \cdot L^{-1}$)	VS ($g \cdot L^{-1}$)	TCH ($g \cdot L^{-1}$)	RS ($g \cdot L^{-1}$)
C-H	2.7	29.04 ± 0.59	23.6 ± 1.05	13.31 ± 0.01	12.16 ± 1.71
TV-H	2.7	21.34 ± 0.14	18.25 ± 0.07	4.55 ± 0.50	1.97 ± 0.01

To calculate the H_2 yield in DF of this BHP test, the mass loss (TS) during the pretreatment stage was determined. **Figure 4.5** displays the mass loss in the substrate, considering the control (C-H) and the *T. versicolor* pretreated substrate after 25 d of SSF (TV-H).

After 25 days of pretreatment under the conditions established in the previous subsection, a mass loss of 30.31 ± 3.79 % was determined in the pretreated substrate. This parameter plays a key role in comprehensively assessing the effects of pretreatment on H_2 yield, since aerobic biomass degradation can redistribute

carbon into different forms, including soluble carbon compounds, fungal biomass (mycelium), and CO_2. Among these, CO_2 cannot serve as a carbon source, and its production represents a direct carbon loss that may negatively affect hydrogen yield. Consequently, one of the main challenges associated with this pretreatment approach is achieving an appropriate balance between lignin removal and the preservation of sugars and organic matter. Nevertheless, when dealing with complex and recalcitrant materials such as lignocellulosic biomass, the mass loss occurring during pretreatment may be compensated by the improved bioaccessibility of the substrate (Brémond et al., 2018a).

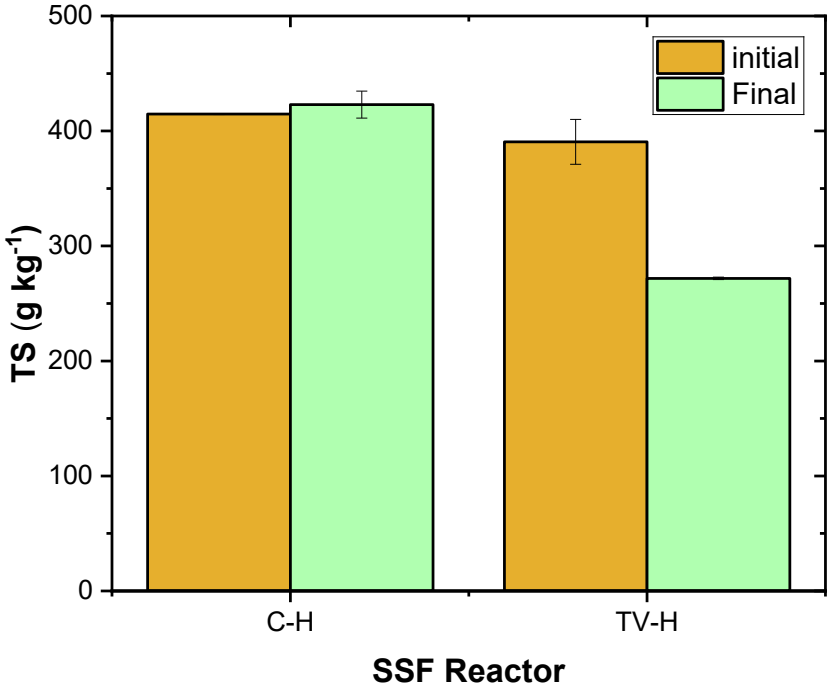


Figure 4.5. Mass loss (TS) observed after fungal pretreatment through SSF using *T. versicolor* for 25 d at 28°C in control (C-H) and *T. versicolor*-pretreated hydrolysate (TV-H).

4.4.1. Hydrogen Production Performance and Physicochemical Analysis of the BHP test reactors

The reaction lasted until H_2 production ceased, after 72 h. **Figure 4.6** displays the initial and final concentration of RS in the two groups tested. It is clear that the C-H group showed a higher RS concentration at the beginning of DF, as no sugar consumption occurred, unlike the case of the TV-H, observing clearly not only lignin degradation but also sugar consumption in the pretreatment stage. RS concentration in the C-H system was approximately 5.5 times higher than the TV-H initial RS concentration. However, at the end of the process, after 72 h, RS consumption was similar in both groups, with values ranging between 87% and 89%, indicating no difference in sugar consumption in the two groups.

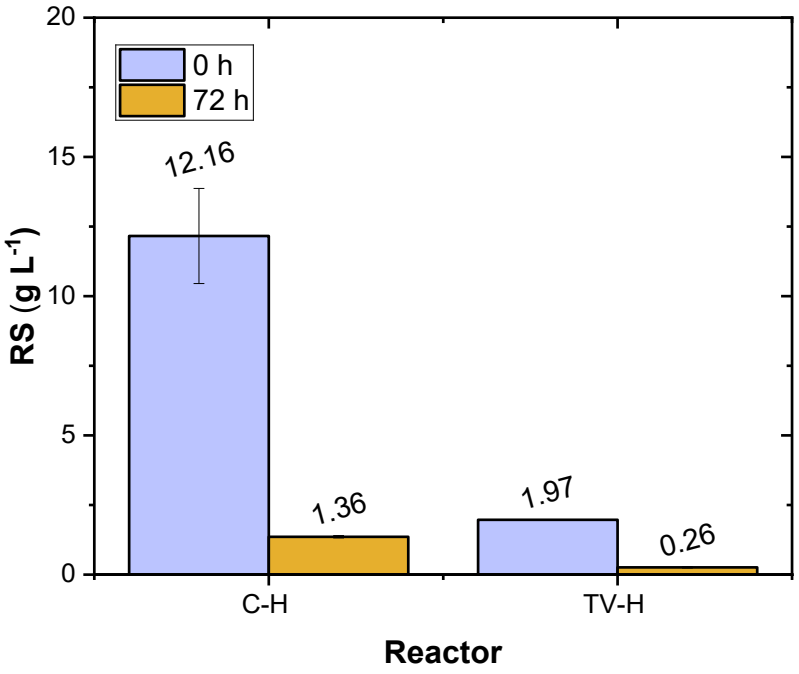


Figure 4.6. Initial and final RS concentrations in both groups of reactors during DF in the BHP test (GAS, 37°C, pH 5.50 ± 0.05, S/I 2.7, 150 rpm).

Despite having a higher initial RS concentration, the control group was the least efficient in terms of cumulative H_2 production at the end of the process, as well as the overall percentage of hydrogen in biogas and hydrogen yield.

Taking this into account, **Figure 4.7a** and **b** present the cumulative hydrogen production and hydrogen content in the biogas, respectively. The control group

achieved only 166 ± 1 $mLH_2 \cdot L^{-1}$ after 72 h and exhibited lower hydrogen percentages in the biogas throughout the sampling times. In contrast, the TV-H group reached a cumulative hydrogen production of 341 ± 1 $mLH_2 \cdot L^{-1}$ and showed the highest hydrogen proportion in the biogas, reaching 66% during the first two sampling times.

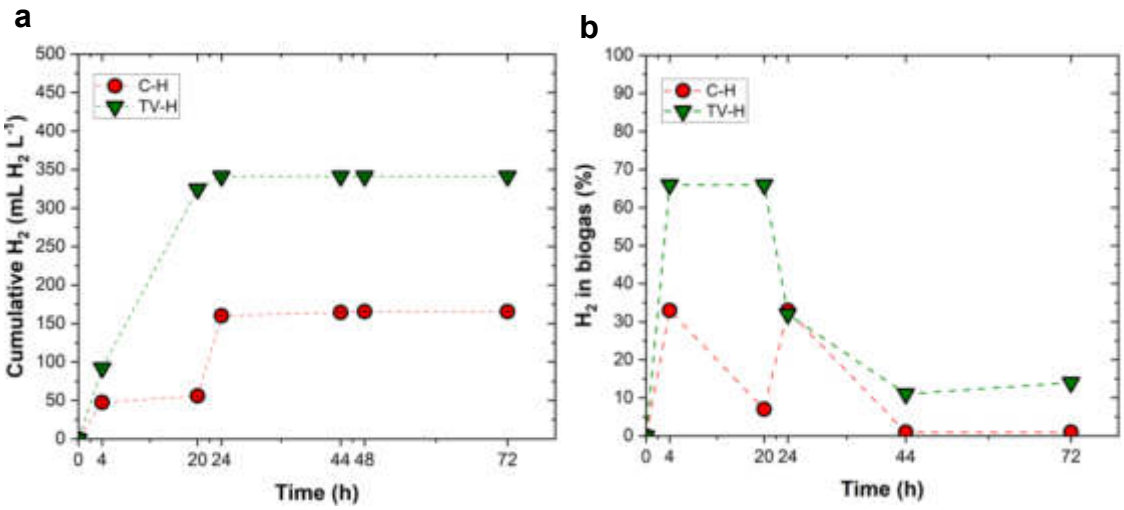


Figure 4.7. (a) Cumulative H_2 production along the DF process in the BHP test after 72 h, and (b) General percentage of H_2 in biogas in the two groups of reactors used in the BHP test (GAS, 37°C, pH 5.50 ± 0.05, S/I 2.7, 150 rpm).

In the present work, however, an opposite trend with respect to other studies where fungal-pretreated substrates were used in biogas production was observed. Such is the case of the results obtained by Mackul'ak et al., 2012, who reported a 15% increase in biogas production when sweet chestnut leaves were pretreated for 4–5 weeks with the wood-decaying fungus *Auricularia auricula-judae* compared to the untreated substrate. Similarly, Panngoen et al., 2023 observed an increase in biogas yield from 82.29 ± 2.22 $mL \cdot gVS^{-1}$ for untreated oil palm kernel pulp to 154.53 ± 0.43 $mL \cdot gVS^{-1}$ after pretreatment with *Pleurotus ostreatus* (30 days, 70 % moisture). In this work, less cumulative biogas production was observed when using fungal-pretreated substrate (517.5 ± 26.5 $mL \cdot L^{-1}$) compared to the control group, where the raw substrate was used (1775.0 ± 70.6 $mL \cdot L^{-1}$) after 72 h of fermentation. Additionally, in the TV-H group a better buffer effect was observed, since the final pH in this group was 5.39 ± 0.01 compared to the C-H group, with a final pH of 4.29 ±

0.06. CO_2 was the dominant gas in the C-H group throughout all sampling times, suggesting the occurrence of alternative metabolic pathways when the untreated substrate was employed, which directly affected H_2 production and the final pH of the system. In contrast, reactors of the TV-H group exhibited a higher proportion of H_2 in biogas. This behavior may be associated with the substantial reduction in lignin content, which enhances the bioaccessibility of reducing sugars contained in the holocellulosic fraction for hydrogen-producing bacteria (HPB). A similar effect was described by Buffiere et al., 2006who reported improved CH_4 production during anaerobic digestion when substrates with lower lignin content were used, attributing this improvement to the greater accessibility and hydrolysis of the substrate during the process.

Although the TV-H group presented a lower initial RS concentration, it achieved greater H_2 production, suggesting that the fungal pretreatment enhanced hydrogen generation, resulting in a 2.06-fold increase compared to the C-H group. This observation is consistent with previous studies where a 1.8-fold improvement in H_2 production relative to untreated controls using wheat straw pretreated with *Phanerochaete* chrysosporium by SSF at 30°C for 12 d was observed, attributing this enhancement to a 29% reduction in lignin content during pretreatment (Ravindran & Jaiswal, 2016). These findings support the idea that the mass loss observed during the pretreatment stage can be compensated by the increased substrate accessibility achieved through delignification.

Nevertheless, hydrogen production reached a stationary phase after approximately 40 h of fermentation. This trend is likely related to the depletion of fermentable sugars within the system, a phenomenon commonly reported in studies involving lignocellulosic substrates. In many cases, the stationary phase of H_2 production is observed within the first 24-48 h of fermentation. For example, Gómez-Camacho et al., 2021 described the cessation of hydrogen production between 33 and 48 h during the fermentation of maize/grass silage, attributing this behavior to substrate depletion and physiological shifts in the acidogenic bacterial community. Likewise, de Vrije et al., 2009 identified a lag phase in hydrogen production between

25 and 40 h when *Miscanthus* hydrolysate was used as substrate, reporting that most sugar consumption and hydrogen production had already occurred within that period.

4.4.2. Kinetic Analysis of H_2 Production

After analyzing the hydrogen production performance of both groups evaluated in the BHP test, the Gompertz model was used to describe and predict the kinetics of hydrogen production over time, as well as to estimate important parameters such as maximum hydrogen production, lag phase duration, and maximum hydrogen production rate. **Table 4.6** displays the hydrogen yield and productivity in C-H and TV-H groups using the Gompertz model, considering, as mentioned above, the mass loss observed during fungal pretreatment in the case of the TV-H group.

Table 4.6. Predicted hydrogen yield, Hmax, Rmax, and lag phase values obtained from the Gompertz model, together with the yield enhancement achieved after considering biomass losses during the pretreatment stage.

Reactor	H_2 Yield ($mLH_2 \cdot g\ VS^{-1}$)	$Hmax$ ($mL \cdot L^{-1}$)	$Rmax$ ($mL \cdot h^{-1}$)	Λ (h)	Mass loss compared to control (% TS)	r^2	Yield improvement considering mass loss (%)
C-H	7	165.08	53.80	18.96	-	0.98	-
TV-H	19	339.65	34.05	1.36	30.40	1.00	43

As shown in the previous table, the C-H group exhibited a higher Rmax value (53.80 $mL \cdot h^{-1}$) than the TV-H group (34.05 $mL \cdot h^{-1}$), which could be associated with the greater RS concentration present in the C-H group. Nevertheless, the TV-H group presented a shorter λ (lag phase) value of 1.36 h, likely as a consequence of the lower lignin content in the pretreated substrate. This reduction in lignin may have facilitated the hydrolysis stage during dark fermentation, promoting a faster onset of hydrogen production.

4.4.3. VFAs Accumulation and Microbial Community Analysis

VFAs production was determined at the end of the DF process. As can be observed in **Figure 4.8**, the main by-products were acetic and butyric acid. Their concentrations were higher in the TV-H group, where a final concentration of 0.108 $g \cdot gVS^{-1}$ of acetic acid, 0.02 $g \cdot gVS^{-1}$ of butyric acid, and 0.005 $g \cdot gVS^{-1}$ of propionic acid was produced. In the C-H group, on the other hand, a final concentration of 0.03 $g \cdot gVS^{-1}$ of acetic acid, 0.003 $g \cdot gVS^{-1}$ of butyric acid, and 0.001 $g \cdot gVS^{-1}$ of propionic acid was observed. Both groups of reactors exhibited similar acetate-to-propionate ratios, indicating that the acetate pathway predominated regardless of the pretreatment. Since propionate formation is considered a hydrogen-consuming pathway, the consistently high acetate/propionate ratio suggests that both systems presented a limited hydrogen loss through the propionate pathway, as only trace amounts of this VFA were observed (Bernardo Ruggeri, Tonia Tommasi, 2015).

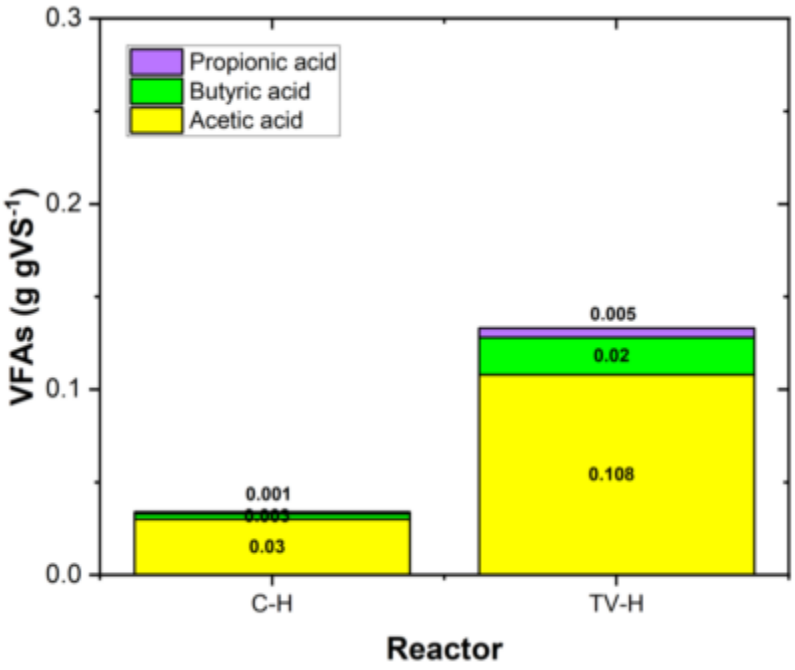


Figure 4.8. Cumulative VFA production after 72 h of fermentation in the BHP test (GAS, 37°C, pH 5.50 ± 0.05, S/I 2.7, 150 rpm).

Acetic acid as a coproduct can be associated with H_2 production through the acetate pathway (**Equation 4.1**), however, its presence can also suggest the occurrence of homoacetogenesis (**Equation 4.2**), a pathway in which the hydrogen

generated during fermentation is consumed for acetic acid formation (X. M. Guo et al., 2014). For this reason, the Hydrogen Production Efficiency (HPE) was determined for both reactor groups, using **Equation 3.4**. An HPE value of 1 indicates that acetic acid is formed solely via the acetate pathway (**Equation 4.1**), whereas values below 1 suggest that a fraction of the acetate is produced through homoacetogenesis (**Equation 4.2**). In both reactor groups, HPE values varied between 0.21 and 0.30, revealing partial hydrogen consumption under both conditions. Nevertheless, the TV-H group showed a greater concentration of butyric acid, which, unlike acetic acid, is considered a direct indicator of hydrogen generation through the butyrate pathway (**Equation 4.3**). This behavior may account for the higher H_2 accumulation observed in this experimental group (Dauptain et al., 2024).

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \textbf{Equation 4.1}$$

$$4H_2 + 2CO_2 \longrightarrow CH_3COOH + 2H_2O \qquad \textbf{Equation 4.2}$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \textbf{Equation 4.3}$$

The biological pretreatment using *T. versicolor* applied to the substrate used in TV-H group not only improved hydrogen yield and production but also enhanced cumulative VFA production by altering the metabolic pathways compared to the C-H group. This is related to the bacterial communities present in both groups at the end of DF, as shown in **Figure 4.9**, as distinct bacterial OTUs and different percentages of relative abundance were observed in each group.

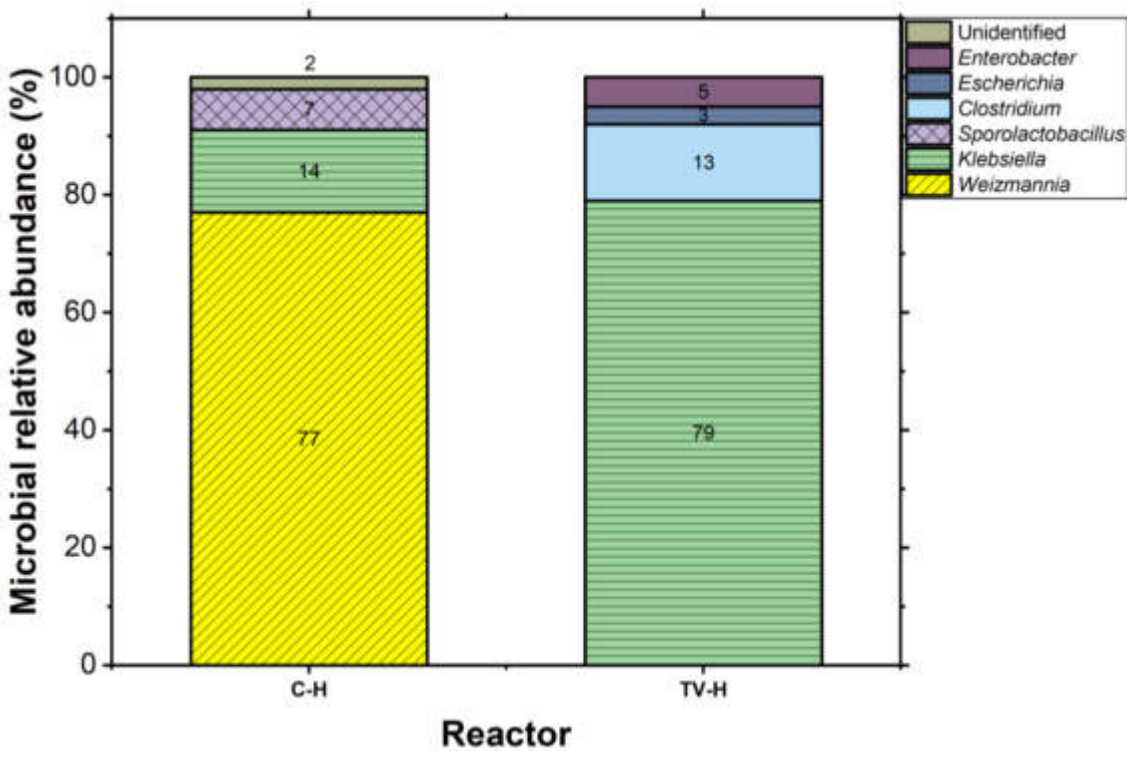


Figure 4.9. Microbial relative abundance at the end of DF in both groups studied in the BHP test (GAS, 37°C, pH 5.50 ± 0.05, S/I 2.7, 150 rpm).

Distinct microbial communities were observed in each experimental group. In the C-H group, the dominant OTU corresponded to the genus *Weizmania*, representing 77% of the microbial population at the end of the process, followed by *Klebsiella* with a relative abundance of 14%. Although members of the genus *Weizmannia* have not previously been associated with H_2 production through dark fermentation, recent studies have reported their capacity to produce lignocellulolytic enzymes and degrade complex carbohydrates (Aulitto et al., 2022; Pamueangmun et al., 2023). Therefore, their presence in the C-H group could be attributed to their involvement in complex substrate hydrolysis, facilitating the degradation of the lignocellulosic structure. On the other hand, in the TV-H group, the presence of *Weizmannia* appeared to be less relevant, since the substrate had already undergone degradation through the enzymatic activity of *T. versicolor*. As a result, the microbial community in the TV-H system shifted toward a greater abundance of HPB. These included OTUs related to the *Klebsiella* (79%) and *Clostridium* genera

(13%) by the end of fermentation. Overall, these findings indicate that fungal pretreatment of the lignocellulosic substrate markedly modifies the microbial community structure during subsequent dark fermentation, promoting the enrichment of *Klebsiella* and *Clostridium* genera while increasing OTU diversity within the latter group.

Even though *Klebsiella* species are non-spore-forming and non-thermophilic bacteria, the presence of non-spore-forming microorganisms in DF systems after the application of thermal pretreatments to the inoculum has been previously reported (Reyna-Gómez et al., 2021). This phenomenon has been associated by several authors with the persistence of DNA from cells that were inactivated during substrate and/or inoculum pretreatment (F. Guo & Zhang, 2014). Nevertheless, the detection of these microorganisms may also be influenced by the characteristics of the inoculum employed. In this study, granular anaerobic sludge was used, and the granular structure may provide physical protection to certain microorganisms against thermal inactivation. Milferstedt et al., 2017 reported that these granules contain a matrix of extracellular polymeric substances (EPS) capable of generating microenvironments that regulate diffusion processes and reduce physical stress. In addition, the authors observed that hydrogen-producing bacteria (HPB) can produce between 2 and 10 times more EPS than methanogenic microorganisms. This may explain the proliferation of *Klebsiella* in the reactor, as well as the absence of methanogens during DF.

Although only a limited number of studies have evaluated the effect of fungal pretreatment on the substrate, the results obtained in this study are in agreement with those reported by H. Zhang et al., 2022 who observed enhanced hydrogen production when using coal modified through pretreatment with the WRF *Phanerochaete chrysosporium*. The authors reported a greater proliferation of *Clostridium* bacteria in the fungal-pretreated substrate compared to the untreated material. On the other hand, Dauptain et al., 2024 evaluated the influence of acid pretreatment applied to the biomass substrate on metabolic pathways and bacterial community composition. Their results showed that the use of pretreated substrate

promoted changes in the microbial community, mainly characterized by the proliferation of *Enterobacteriales* and *Clostridiales*. The authors associated this behavior with the partial hydrolysis of the substrate caused by the pretreatment, which also modified the fermentation byproducts, leading to a shift from acetate production in the control group toward butyrate and ethanol formation when the pretreated substrate was employed. The variation observed in bacterial community composition directly influences by-product formation, since the metabolic pathways involved in the process are governed by microbial activity. These differences in microbial populations may be associated with the physical and chemical modifications generated in the substrate during pretreatment. In particular, the reduction in lignin content during this stage likely increased sugar bioaccessibility, thereby favoring the selective proliferation of specific bacterial groups (Agosin et al., 1985; Rouches et al., 2016).

A marked predominance of *Klebsiella* and *Clostridium* was identified in the TV-H group, both of which are extensively recognized for their H_2-producing capabilities (Jamaldheen et al., 2022; Niu et al., 2010; Tapia-Venegas, Ramirez-Morales, et al., 2015; H. Zhang et al., 2006). The higher hydrogen content in the biogas, together with the enhanced hydrogen yield and the greater cumulative H_2 production observed at the end of fermentation in this group, may be associated with the proliferation of these bacterial genera. Their enrichment was likely promoted by the physicochemical modifications generated in the substrate following pretreatment with *Trametes versicolor*.

In a study conducted by Niu et al., 2010 *Klebsiella pneumoniae*, which was the predominant bacterial genus identified in the TV-H group, exhibited the highest hydrogen production rate. However, the authors reported that its H_2 yield remained lower than that achieved by other genera, such as *Clostridium* and *Enterobacter*, both of which were also detected in the TV-H group.

It is widely recognized that *Clostridium* species are among the main bacterial genera responsible for H_2 production through DF, particularly when microbial consortia derived from wastewater treatment plants or rumen are used as inoculum

(Bernardo Ruggeri, Tonia Tommasi, 2015). Accordingly, *Clostridium* accounted for 13 % of the bacterial OTUs identified in the TV-H group. This likely had a direct impact on hydrogen yield, as well as on butyric and acetic acid production, since the metabolism of *Clostridium* species is associated with the formation of these metabolites and hydrogen gas (Bernardo Ruggeri, Tonia Tommasi, 2015; Noguer et al., 2022).

CHAPTER 5

5. Results in the modified BHP test and BMP test

The experimental work and results displayed in this chapter were carried out at the LBE facilities in Narbonne, France. Thus, the fungal pretreatment in this chapter as well as in Chapter 6 was carried out with *T. versicolor* CIRM-BRFM 277 (Marseille, France) in place of *T. versicolor* TV-38 from Fungi MTY.

5.1. Modified BHP test

Once the H_2 production potential of the raw substrate and the substrate pretreated with *T. versicolor* for 25 days at 28°C had been determined, a modification of the BHP test was carried out with the aim of improving H_2 production. Although a 43% increase in H_2 yield was observed when the substrate pretreated by SSF was used, the yield remained considerably low (18.70 $mLH_2 \cdot gVS^{-1}$) compared to the values reported in the literature when lignocellulosic substrates are used (Manuel et al., 2022; Martínez-Fraile et al., 2024).

For this purpose, the second lignocellulosic mixture described in **Section 3.1** was employed, modifying the process conditions established in **Section 3.4.2**.

5.1.1. Hydrogen Production Performance and Physicochemical Analysis of the Modified BHP Test Reactors

H_2 production was evaluated again in two different groups: raw substrate (RAW MIX) and substrate pretreated for 25 days (SSF25d MIX). **Table 5.1** displays the physicochemical characteristics of each reactor at the beginning of DF in the modified BHP test. As can be observed, the initial physicochemical characteristics of both reactor groups revealed important differences in RS and VS concentrations. The SSF25d MIX group again exhibited a lower RS concentration (0.06 ± 0.01 $g \cdot L^{-1}$) compared to the RAW MIX group (1.72 ± 0.22 $g \cdot L^{-1}$), a difference attributed to the metabolic activity of *T. versicolor* during pretreatment. However, as observed in the previous BHP test, this reduction in fermentable sugars does not necessarily represent a detrimental effect on hydrogen production, since WRF pretreatment promotes delignification and structural disruption of lignocellulosic biomass, thereby enhancing cellulose accessibility and enzymatic digestibility during the DF process (Suryadi et al., 2022; Yoav et al., 2018).

Table 5.1. Initial physicochemical characteristics in both reactor groups studied in the modified BHP test (37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

System	RS ($g \cdot L^{-1}$)	VS ($g \cdot L^{-1}$)	pH
RAW MIX	1.72 ± 0.22	8.38 ± 0.34	5.58 ± 0.01
FP25D MIX	0.06 ± 0.01	1.49 ± 0.04	5.58 ± 0.04

As observed in the previous BHP test, the enhanced accessibility of the substrate may offset the initial depletion of sugars during the pretreatment stage. This effect is associated with lignin degradation and alterations in the cell wall matrix, which reduce biomass recalcitrance and promote the hydrolysis of structural carbohydrates during DF (Rouches et al., 2016). Nevertheless, the consequences of this balance between RS depletion and increased accessibility depend on the parameter used to evaluate the DF process. Although a lower initial RS concentration may reduce the total amount of H_2/biogas generated because of the lower availability of readily fermentable sugars, the greater accessibility of structural carbohydrates can improve substrate conversion efficiency. This difference is particularly important when comparing cumulative H_2 production and H_2 yield, since the latter (expressed per unit of VS) may increase even when the overall hydrogen production decreases.

Building upon the previously discussed trade-off, hydrogen production in the modified BHP test was evaluated in terms of cumulative hydrogen production ($mL \cdot L^{-1}$) and H_2 yield ($mL \cdot gVS^{-1}$) to better distinguish between total output and substrate conversion efficiency. In terms of cumulative hydrogen production, **Figure 5.1** shows that, in this case and unlike the first BHP test, the RAW MIX group, in which the untreated substrate was used, exhibited greater hydrogen accumulation. This behavior is consistent with the higher concentration of reducing sugars present in this group. In contrast, the SSF25d MIX group showed lower cumulative H_2 production throughout the fermentation process, reaching a stable stationary phase at 42 h of reaction. This behavior may be closely associated with the low initial reducing sugar concentration in the SSF25d MIX group, which was only 0.06 ± 0.01 $g \cdot L^{-1}$. By comparison, the TV-H group evaluated in the first BHP test under the previously established conditions presented an initial RS concentration of 1.97 ±

0.01 g·L^{-1}, corresponding to a concentration 33 times higher than that observed in the SSF25d MIX group used in this modified BHP test.

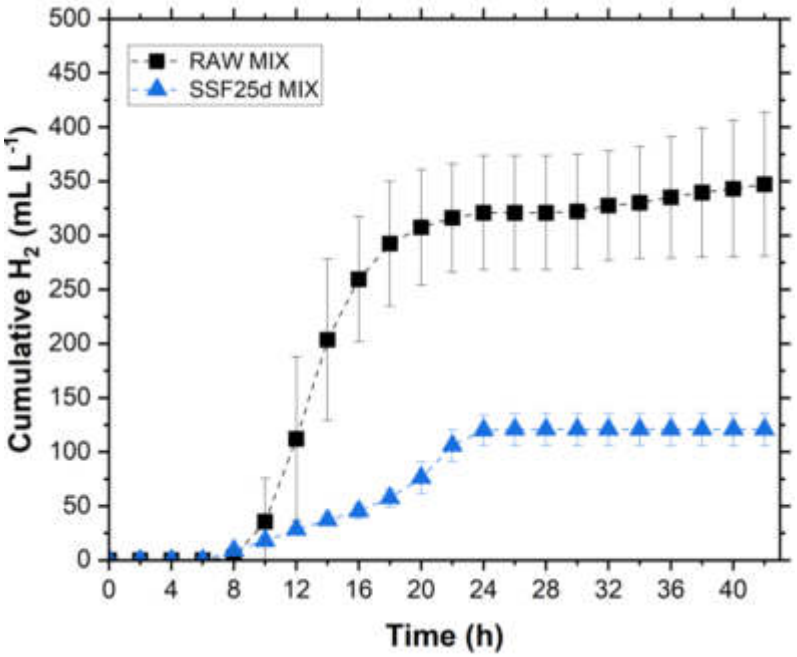


Figure 5.1. Cumulative H_2 production along the DF process in the modified BHP test after 42 h in the two groups of reactors tested: Raw mixture (RAW MIX) and 25-day fungal-pretreated mixture (SSF25d MIX) (AS, 37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

Although lower cumulative hydrogen production was observed at the end of the process in the SSF25d MIX group (121 ± 15 mL·L^{-1}) compared to the RAW MIX group (347 ± 66 mL·L^{-1}), a different trend emerged when hydrogen production was normalized according to the initial volatile solids content of the reactors. H_2 yield, expressed as mLH_2·gVS^{-1}, was higher in the SSF25d MIX group, suggesting that, despite the lower cumulative hydrogen production, pretreatment with *T. versicolor* through SSF improved substrate conversion efficiency by increasing sugar accessibility, as previously observed in the first BHP test. The results obtained in the modified BHP test emphasize the importance of selecting an appropriate performance indicator, since conclusions regarding process efficiency may vary considerably depending on whether cumulative hydrogen production or hydrogen yield is evaluated. Similar observations were reported by Herrmann et al., 2011 who

found organic matter losses ranging from 1 % to 13 % after applying different biological pretreatments based on ensiling with bacterial additives (*Lactobacillus plantarum* and *Lactobacillus buchneri*) to maize, sorghum, forage rye, and triticale prior to CH_4 production through anaerobic digestion. Despite these losses, methane yield increased by up to 17 %, whereas only minor or negligible differences were detected in methane production per unit of initial biomass.

Although H_2 accumulation in the SSF25d MIX group (121 ± 15 $mL \cdot L^{-1}$) evaluated in the modified BHP test was lower than that observed in its counterpart from the first BHP test (TV-H, 341 ± 1 $mL \cdot L^{-1}$), the hydrogen yield obtained in the SSF25d MIX group was higher, reaching 81 ± 9 mL $H_2 \cdot gVS^{-1}$. This value represented a hydrogen yield 4.26 times greater than that obtained in the TV-H group, 11.57 times higher than the raw substrate evaluated in the first BHP test (C-H), and 1.93 times greater than the RAW MIX group analyzed in the modified BHP test (42 ± 7 $mLH_2 \cdot gVS^{-1}$).

Figure 5.2 presents a comparative analysis of the H_2 yield obtained in the four reactor groups evaluated during the first BHP test (C-H = 7 ± 1 $mLH_2 \cdot gVS^{-1}$; TV-H = 19 ± 8 $mLH_2 \cdot gVS^{-1}$) and the modified BHP test (RAW MIX = 42 ± 7 $mLH_2 \cdot gVS^{-1}$; SSF25d MIX = 81 ± 9 $mLH_2 \cdot gVS^{-1}$). As can be observed, in terms of hydrogen yield, pretreatment with *T. versicolor* was effective under both evaluated conditions, improving system performance after modifying the operational variables established in the modified BHP test and achieving a maximum yield of 81 ± 9 $mLH_2 \cdot gVS^{-1}$ in the SSF25d MIX group. These findings indicate that fungal pretreatment with *T. versicolor* under the conditions described in **Section 4.3** substantially improves the efficiency of bioconversion of these lignocellulosic waste mixtures into hydrogen, even achieving higher yields than those reported in some studies using lignocellulosic residues as substrate. For example, Cieciura-Włoch et al., 2020 evaluated hydrogen production using a mixture of fruit and vegetable residues under mesophilic conditions and reported a hydrogen yield of 52 $mLH_2 \cdot gVS^{-1}$, which was lower than the yield observed in the SSF25d MIX group of the modified BHP test.

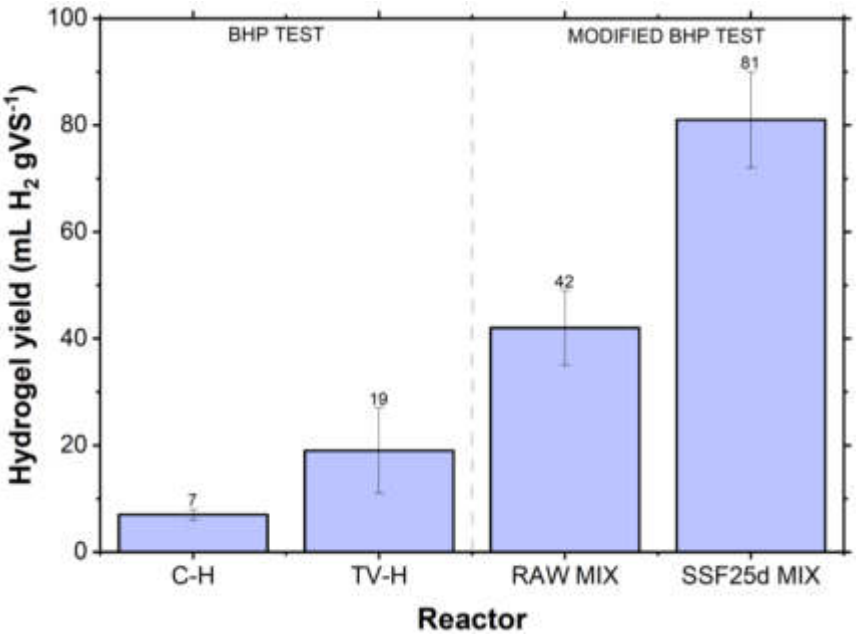


Figure 5.2. Comparative in H_2 yield observed in both BHP tests carried out: Control hydrolysate (C-H) and *Trametes versicolor* hydrolysate (TV-H) from the first BHP test and Raw mixture (RAW MIX) and 25-day fungal-pretreated mixture (SSF25d MIX) from the modified BHP test (AS, 37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

It is important to highlight that this improvement in H_2 yield may also be associated with the nature of the substrate mixture evaluated. The use of mixed substrates generally produces a positive effect on the efficiency of the fungal pretreatment stage, since combining residues with different physicochemical characteristics may generate a complementary effect. In this case, the degradation of readily available sugars derived from fruit peels, together with more recalcitrant lignocellulosic materials such as WS and BSG, may create a more balanced substrate for mycelial colonization and subsequent fermentation during the DF process. Similar synergistic effects have been reported in mixed substrates used for DF, where substrate blending commonly improves nutrient balance, carbon availability, and biodegradability (Mumtha & Mahalingam, 2024; Saha et al., 2022).

Regarding pH, the modified BHP test (RAW MIX and SSF25d MIX) exhibited the same trend observed in the original BHP test, showing a marked buffering effect

in the substrate pretreated with *T. versicolor*. **Figure 5.3** presents the initial and final pH values after 42 h of dark fermentation for both groups evaluated in the modified BHP test. As can be observed, the RAW MIX group reached a final pH of 4.40 ± 0.04, whereas the SSF25d MIX group exhibited a less acidic final pH of 5.04 ± 0.14. This behavior may be associated with the physicochemical modifications induced during fungal pretreatment, including lignin degradation, alteration of functional groups, release of soluble compounds, and a possible enhancement of the buffering capacity of the medium. In addition, the increased accessibility of structural carbohydrates may influence microbial selection and metabolic pathways during DF, potentially favoring bacterial groups commonly associated with butyrate-type fermentation and hydrogen production, which may also influence pH evolution in the pretreated system (J. Sun et al., 2021; X. Sun et al., 2025).

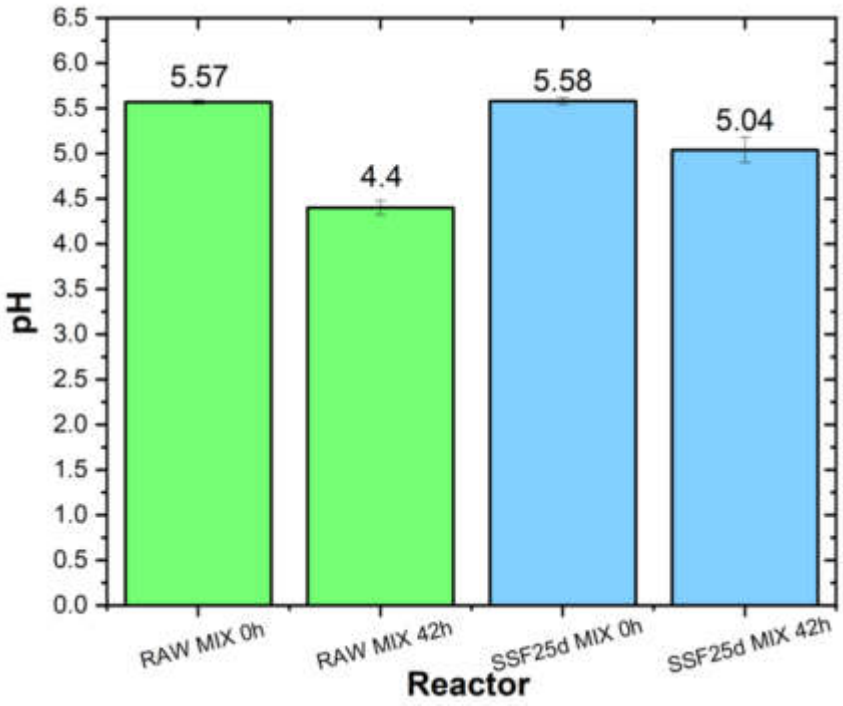


Figure 5.3. Evolution of pH along the DF in the modified BHP test (AS, 37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

5.1.2. VFAs Accumulation and Microbial Community Analysis

Figure 5.4 displays the cumulative concentration of volatile fatty acids produced across the two groups of reactors tested in the modified BHP test (RAW MIX and SSF25d MIX). The samples were taken at the end of the fermentation process after 42 h, resulting in VFA accumulations of 0.037 $g \cdot gVS^{-1}$ of acetic acid and 0.072 $g \cdot gVS^{-1}$ of butyric acid in the RAW MIX, and 0.228 $g \cdot gVS^{-1}$ of acetic acid and 0.201 $g \cdot gVS^{-1}$ of butyric acid in the SSF25d MIX. The VFA profiles observed in the present experimental stage provide important insights into the metabolic pathways involved in H_2 production during the modified BHP test, since only butyrate and acetate were observed in both groups of reactors tested, indicating that hydrogen production mainly proceeded through acetate- and butyrate-type fermentation pathways (**Equations 4.1** and **4.3**). Furthermore, in contrast to the first BHP test, there were no traces of propionic acid, as propionate synthesis requires consumption of H_2 (**Equation 5.1**), its absence indicates that hydrogen losses through this pathway were negligible. This observation agrees with the improved H_2 yields obtained in the modified BHP test assay and suggests that the conditions tested in this experimental stage promoted metabolic routes favorable for H_2 production (Bernardo Ruggeri, Tonia Tommasi, 2015; Lee & Rittmann, 2009).

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O \qquad \textbf{Equation 5.1}$$

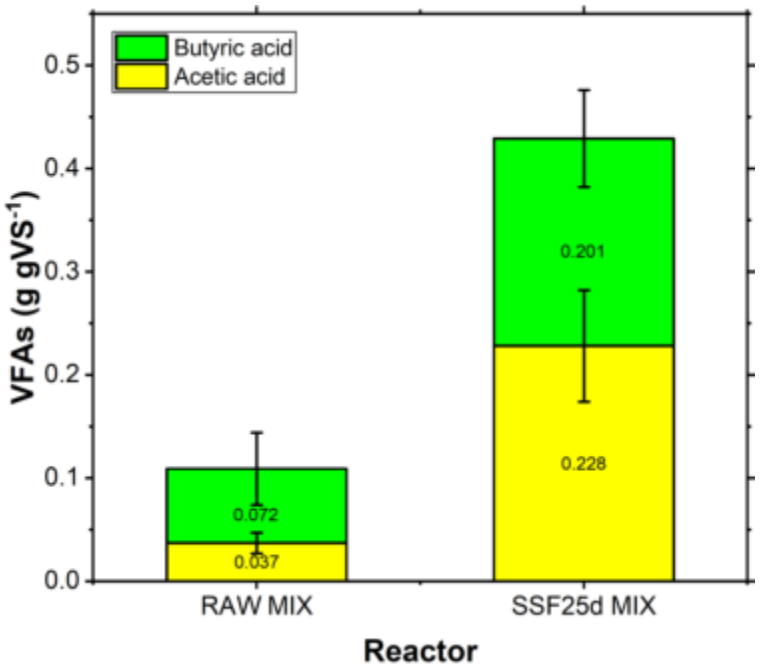


Figure 5.4. Cumulative VFA production after 42 h of fermentation in the modified BHP test (AS, 37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

The fungal-pretreated substrate system again exhibited higher VFA accumulation, with a notable increase in butyric acid concentration compared to the TV-H group (0.201 g gVS-1 vs. 0.020 g·gVS^{-1}) evaluated in the previous BHP test, while an increase in acetic acid concentration was also observed, reaching a value of 0.228 g·gVS^{-1}. In contrast, the RAW MIX group, which showed a lower hydrogen yield, also exhibited lower VFA accumulation (0.109 g·gVS^{-1}), following the same trend observed in the first BHP test performed. This behavior has been previously reported in studies involving pretreatment with WRF. For instance, *Pleurotus sajor-caju* increased VFA production by 60-74% from lignin-rich mushroom residues through selective lignin degradation, thereby improving substrate digestibility during anaerobic fermentation (Fang et al., 2020). Similarly, in another study, they reported an increase of up to 124 % in VFA yield when using anaerobic fermentation digestate pretreated with *P. sajor-caju* compared to the untreated control (Fang et al., 2018). The authors attributed this effect to the increase in substrate surface area and pore size, as well as to delignification and partial hydrolysis generated during fungal pretreatment (Fang et al., 2018, 2020). Based on the above, the HPE index was

calculated again for both groups evaluated, obtaining HPE values of 0.63 for the RAW MIX group and 0.28 for the SSF25d MIX group. These results indicate partial hydrogen consumption for acetic acid production through the homoacetogenesis pathway described in **Equation 4.2**.

The microbial communities developed during the DF process in the modified BHP test varied markedly compared to the microbial relative abundance observed in the previous BHP test, confirming the changes induced by the modified operational conditions applied in this second system. As observed in the previous BHP test, *T. versicolor* pretreatment of the lignocellulosic mixture potentially influences microbial selection in the DF process. **Figure 5.5** presents the microbial relative abundance in both systems studied in the modified BHP test, replicating the trend observed in the previous BHP test, since the proliferation of HPB was favored in the SSF25d MIX group, where a relative abundance of 70% was observed for the OTU associated with the genus *Clostridium*, followed by 9% corresponding to *Klebsiella* and 3% to *Paraclostridium*. Together, these OTUs represented 82% of the total relative abundance of microorganisms associated with hydrogen-producing bacteria. In contrast, the RAW MIX group exhibited a relative abundance of 60% for the OTU related to *Clostridium* and 5% for *Enterobacter*, resulting in a total HPB relative abundance of 65%. In addition, the second most abundant OTU identified in this group corresponded to bacteria of the genus *Lactobacillus*, with a relative abundance of 19%. The high relative abundance of this latter OTU may indicate competitive substrate consumption, diverting the process away from H_2-producing pathways and potentially limiting overall H_2 yields (Correa-Villa et al., 2024). Therefore, shifts in microbial communities favoring *Clostridium* over *Lactobacillus* and other non-HPB may partially explain the enhanced fermentative H_2 performance observed in the SSF25d MIX group after fungal pretreatment (Bernardo Ruggeri, Tonia Tommasi, 2015; Gupta et al., 2024).

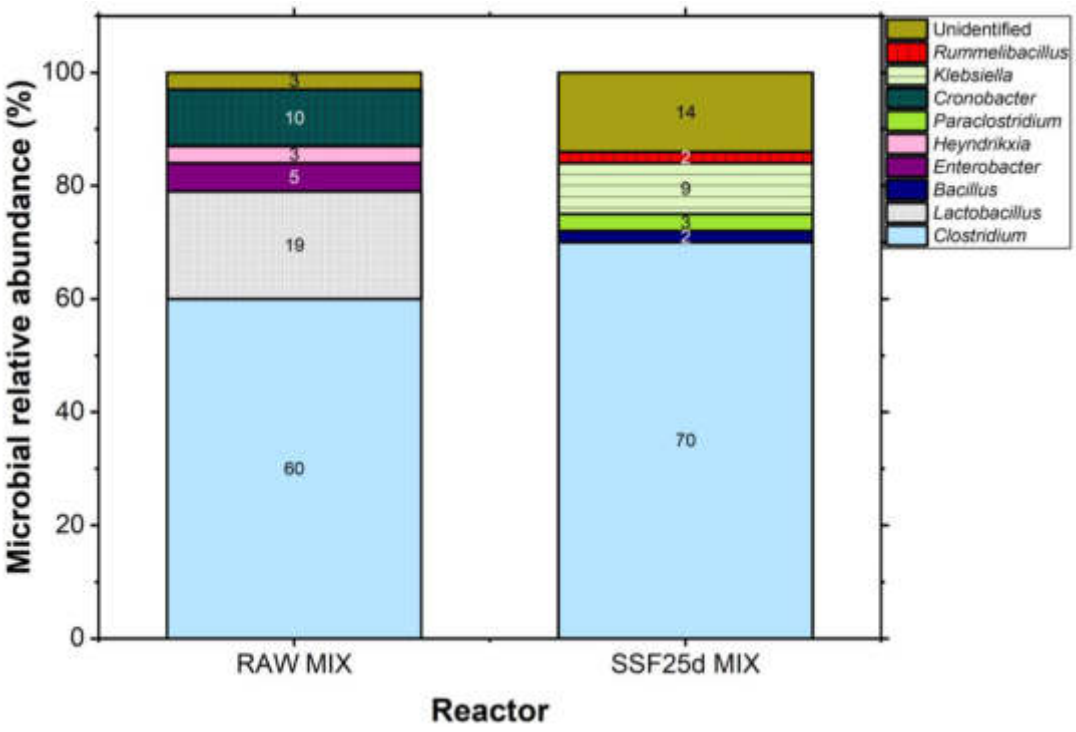


Figure 5.5. Microbial relative abundance at the end of DF in both groups studied in the modified BHP test (AS, 37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

The changes in microbial communities observed in both groups were consistent with the metabolic profiles previously obtained for VFA production. For instance, in the SSF25d MIX system, the enrichment of OTUs associated with the genus *Clostridium* explains the increased concentrations of acetic and butyric acids observed at the end of the process, in agreement with the known metabolic capabilities of this genus. Additionally, the absence of *Lactobacillus*-related OTUs in this system may explain the higher hydrogen yield obtained (Romo-Hernández & Vital-Jácome, 2025).

As previously mentioned, *Clostridium* spp. have been widely reported in efficient DF systems because of their high capacity for H_2 production. During Clostridial-type fermentation, pyruvate ferredoxin oxidoreductase (PFOR) catalyzes the oxidation of pyruvate into acetyl coenzyme A (acetyl-CoA) in the presence of ferredoxin, which becomes reduced during the reaction. Likewise, NADH: ferredoxin oxidoreductase (NFOR) mediates the reduction of ferredoxin using NADH as an

electron donor. The electrons released from reduced ferredoxin are subsequently utilized by hydrogenases for proton reduction and hydrogen generation (Gupta et al., 2024). These changes indicate that fungal pretreatment modified the physicochemical characteristics of the substrate sufficiently to alter the ecological niches available during DF. The partial hydrolysis generated during fungal pretreatment with *T. versicolor* likely modified carbohydrate accessibility and composition, directly influencing the subsequent DF process, since hydrogen production and microbial community structure have been reported to depend on sugar type and degree of polymerization (Chatellard et al., 2016).

Additionally, the presence of another HPB was detected in higher proportions exclusively in the systems pretreated with *T. versicolor* in both BHP tests evaluated: *Klebsiella*. Certain *Klebsiella* species, such as *K. pneumoniae*, have been previously reported as hydrogen-producing bacteria. These microorganisms generate hydrogen through a mixed-acid fermentation pathway, converting glucose and other sugars into different fermentation end products. In this process, formate produced via the formate hydrogen lyase (FHL) pathway is cleaved by the FHL complex through the activity of formate dehydrogenase and hydrogenase, leading to the production of H_2 and CO_2 (Gupta et al., 2024; Liu & Fang, 2007; Niu et al., 2010). Although *Klebsiella* does not exhibit the same hydrogen-producing capacity as *Clostridium*, this bacterial genus has also been successfully employed for hydrogen production in dark fermentation systems (Niu et al., 2010). The overall increase in acetic and butyric acid concentrations observed in the fungal-pretreated systems, where the presence of *Clostridium* and *Klebsiella* was higher, suggests a shift toward acidogenic fermentative pathways commonly associated with hydrogen-producing consortia. The predominance of *Clostridium* likely promoted both acetate- and butyrate-type fermentation pathways, which are widely recognized as the main hydrogen-producing routes during DF. Meanwhile, *Klebsiella* may have contributed to acetate production and complementary fermentative metabolism within the microbial consortium (D'Silva et al., 2023; Ghimire et al., 2015; Show et al., 2012).

5.2. BMP test and biodegradability determination of raw and pretreated mixture

After evaluating the results obtained in the modified BHP test in terms of hydrogen yield, VFAs production, and microbial community composition, and confirming that these parameters were effectively enhanced compared with the original BHP tests, a BMP test was conducted to determine the biodegradability of the second lignocellulosic mixture used in the modified BHP test.

Table 5.2 summarizes the COD and VS values, as well as the corresponding COD/VS ratios obtained for both substrates.

As expected, the RAW MIX exhibited higher COD and VS concentrations, reaching 1.630 and 1.523 $g \cdot g_{Biomass}^{-1}$, respectively. These values were approximately 19.5-fold higher than those obtained for the SSF25d MIX, which presented COD and VS concentrations of 0.08 and 0.09 $g \cdot g_{Biomass}^{-1}$, respectively. A similar trend was observed during the BHP assays in terms of RS and VS, where the untreated substrates consistently exhibited higher concentrations than the substrates pretreated with Trametes versicolor. This behavior can be attributed to the consumption of organic matter during the fungal pretreatment stage, resulting in a reduction of the organic fraction available in the pretreated materials (Aguiar et al., 2014).

Table 5.2. Physicochemical characterization of raw and fungal-pretreated substrate used in the BMP test.

Substate	COD ($g \cdot g_{Biomass}^{-1}$)	VS ($g \cdot g_{Biomass}^{-1}$)	COD/VS
RAW MIX	1.630	1.524	1.07
SSF25d MIX	0.084	0.090	0.93

The COD/VS ratio of the raw mixture (1.07) was within the range reported for lignocellulosic agricultural residues. Camarena-Martínez et al., 2020 reported values of 0.79-0.86 $gCOD \cdot gVS^{-1}$ for tomato and pepper plant residues, while approximately 1.2 $gCOD \cdot gVS^{-1}$ has been reported for wheat straw. Therefore, the obtained value is consistent with the heterogeneous lignocellulosic composition of the substrate.

The decrease to 0.93 $gCOD \cdot gVS^{-1}$ after fungal pretreatment suggests partial consumption of organic matter during fungal growth rather than a deterioration of substrate quality.

After physicochemical characterization of the prepared solutions used to determine the COD/VS ratio, the BMP assay was conducted using the RAW MIX and the SSF25d MIX in triplicate. Additionally, a control (CTRL) containing only the inoculum was included to determine its endogenous methane production. **Figure 5.6** shows the cumulative methane production observed in the three groups set up throughout 40 d of reaction. As can be observed, RAW MIX exhibited a higher cumulative CH_4 production compared with the SSF25d MIX (884.92 ± 35.80 and 709.92 ± 56.19 $mL \cdot L^{-1}$, respectively) following the same trend observed in the modified BHP test, where higher cumulative H_2 was observed in the RAW MIX due to a higher RS and VS concentration which allows higher biogas production, impacting directly H_2, and, in this case, CH_4 accumulation. Regarding the CTRL group, cumulative CH_4 production reached 140.75 ± 9.38 $mL \cdot L^{-1}$ after 40 d of reaction, representing around 16-20% of the methane produced in the RAW MIX and SSF25d MIX, respectively.

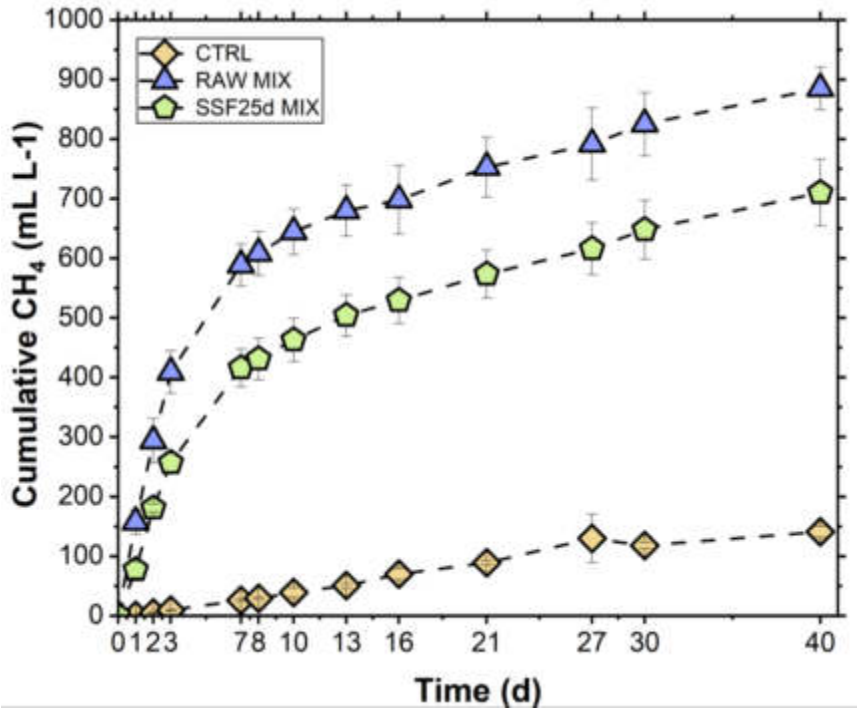


Figure 5.6. Cumulative CH_4 production during 40 d of reaction in the BMP test using the control group of reactors containing only water and inoculum (CTRL), the raw mixture (RAW MIX), and the 25-day fungal-pretreated mixture (SSF25d MIX) (GAS, 35°C, 400 mL WV, S/I 0.5, initial VS 3 $g \cdot L^{-1}$, 150 rpm).

Methane yield was evaluated in the three experimental groups (**Figure 5.7**). The control group exhibited the lowest methane yield, reaching only 40.23 ± 2.75 mL $CH_4 \cdot gVS^{-1}$, followed by the SSF25d MIX with 197.77 ± 10.14 $mLCH_4 \cdot gVS^{-1}$, while the RAW MIX achieved the highest methane yield among the studied groups, reaching 258.73 ± 5.08 $mLCH_4 \cdot gVS^{-1}$.

These results differ from those reported in studies involving lignocellulosic biomass, such as the co-digestion of cow manure and cereal crop residues, where methane yield improvements ranging from 10 to 18% were observed following biological pretreatment with *Trametes versicolor* (Akyol et al., 2019). Nevertheless, the methane yield obtained in the present study was higher than values commonly reported in the literature for anaerobic digestion of similar lignocellulosic substrates. For instance, (López-Aguilar et al., 2025) evaluated the anaerobic co-digestion of

BSG and cattle manure and reported a maximum methane yield of 67.33 $mLCH_4 \cdot gVS^{-1}$.

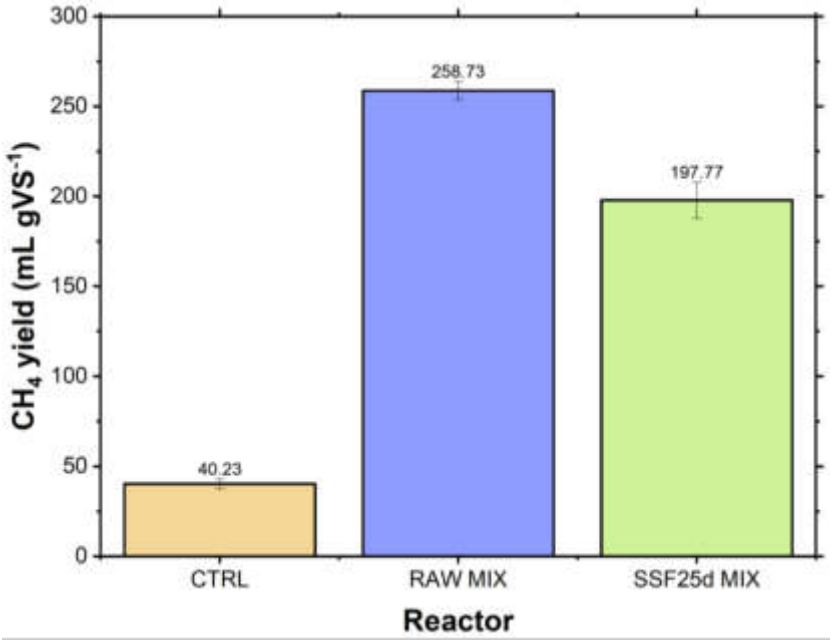


Figure 5.7. CH_4 yield obtained after 40 d of reaction in the BMP test using the control group of reactors containing only water and inoculum (CTRL), the raw mixture (RAW MIX), and the 25-day fungal-pretreated mixture (SSF25d MIX). (GAS, 35°C, 400 mL WV, S/I 0.5, initial VS 3 $g \cdot L^{-1}$, 150 rpm).

Following these results, **Equations 3.5**, **3.6**, **3.7**, and **3.8** were applied to determine the COD added (g), net CH_4 volume (L), biodegradable COD (g), and biodegradability (%), respectively. **Table 5.3** summarizes the values obtained for each parameter in both evaluated substrates.

Table 5.3. Parameters determined after BMP test (GAS, 35°C, 400 mL WV, S/I 0.5, initial VS 3 $g \cdot L^{-1}$, 150 rpm).

Substrate	COD added (g)	Vol CH_4 net ($mL \cdot L^{-1}$)	Biodegradable COD (g)	Biodegradability (%)
RAW MIX	3.21	744.17	0.764	23.80
SSF25d MIX	2.79	569.17	0.585	20.99

The COD added was calculated as 3.21 g for the RAW MIX and 2.79 g for the SSF25d MIX. Regarding net methane production, values of 744.17 $mLCH_4 \cdot L^{-1}$ (0.298 L) and 569.17 $mLCH_4 \cdot L^{-1}$ (0.228 L) were obtained for the RAW MIX and SSF25d MIX, respectively. These methane volumes corresponded to biodegradable COD values of 0.764 and 0.585 g, respectively. Finally, using the calculated parameters, the biodegradability of each substrate was determined. The RAW MIX exhibited a biodegradability of 23.80%, whereas the SSF25d MIX showed a biodegradability of 20.97%.

Although the biodegradability percentage did not improve, showing very similar values between the RAW MIX and the SSF25d MIX, the biological pretreatment with *Trametes versicolor* may have improved sugar bioavailability. As observed in **Sections 4.4.1** and **5.5.1**, related to the BHP tests, the hydrogen yield per gram of initial VS was consistently higher in the pretreated substrates (C-H = 7 ± 1 $mLH_2.gVS^{-1}$ < TV-H = 19 ± 8 $mLH_2.gVS^{-1}$, and RAW MIX = 42 ± 7 $mLH_2.gVS^{-1}$ < SSF25d MIX = 81 ± 9 $mLH_2.gVS^{-1}$). Additionally, in both BHP tests, the concentrations of acetic acid and butyric acid were consistently higher than those observed in the untreated substrate, indicating a greater utilization of the substrate when it was pretreated.

The increase in acetate, butyrate, and hydrogen yield suggests that pretreatment enhanced the accessibility of structural carbohydrates to fermentative microorganisms. However, this improvement in bioavailability did not translate into an increase in the overall biodegradability determined through the BMP assay, probably due to the consumption of organic matter during solid-state fermentation with *T. versicolor*, which reduced the total amount of substrate available (RS) for subsequent anaerobic digestion (Dauptain et al., 2024b; Galbe & Wallberg, 2019).

CHAPTER 6

6. Results in 3 L reactors

6.1. Setup of the 3 L Batch Reactors

After determining the H_2 production potential of both mixtures evaluated under the previously established conditions, the mixture and operational conditions that resulted in the highest H_2 yield and VFA accumulation were selected to replicate these conditions in 3 L batch reactors with 2 L of working volume.

For this purpose, the second mixture evaluated in the modified BHP test was selected under the same operational conditions used in that assay: initial pH of 5.50 ± 0.08, S/I ratio of 20, thermally pretreated aerobic sludge (30 min at 100 °C) used as inoculum, agitation speed of 150 rpm, initial VS concentration below 10 $g \cdot L^{-1}$, and mesophilic conditions (37 °C).

For this experimental stage, controlled pH conditions were maintained throughout the dark fermentation process, preventing the intrinsic acidification of the medium. Two different batch reactors were set up: using raw substrate (RBR) and fungal pretreated substrate (FPBR). The fungal pretreatment was carried out under the same conditions established in **Section 4.3**. The operation of both 2 L batch reactors was carried out for 48 h for both the RBR and the FPBR systems. Likewise, physicochemical parameters such as RS, TS, and VS were evaluated at the beginning of the process, and microbial community composition at the end of the fermentation, after 48 h.

6.1.1. Hydrogen Production Performance and Physicochemical Analysis of the 3 L batch reactors

H_2 production was monitored over the 48 h the process lasted in both reactors: RBR and FPBR. **Table 6.1** shows the initial physicochemical characteristics of each reactor. As can be observed, the concentrations of both RS and solids were again markedly different between systems, with the FPBR exhibiting only 0.69 ± 0.01 $g \cdot L^{-1}$ of RS, 4.20 ± 0.01 $g \cdot L^{-1}$ of TS, and 4.09 ± 0.01 $g \cdot L^{-1}$ of VS.

The RS concentration in the FPBR was five times lower than that of its RBR counterpart (0.69 ± 0.01 vs. 3.51 ± 0.25 $g \cdot L^{-1}$), which could negatively impact biogas production and, consequently, cumulative hydrogen production ($mL \cdot L^{-1}$), as previously observed in the modified BHP test. A similar trend was observed for TS and VS concentrations, with the FPBR presenting approximately three times lower concentrations compared to the RBR system.

Table 6.1. Initial physicochemical characteristics in both batch reactors studied (AS, 37°C, pH 5.50 ± 0.08, S/I 20, 150 rpm).

System	RS ($g \cdot L^{-1}$)	TS ($g \cdot L^{-1}$)	VS ($g \cdot L^{-1}$)
RBR	3.51 ± 0.25	12.52 ± 1.15	11.40 ± 1.17
FPBR	0.69 ± 0.01	4.20 ± 0.21	4.09 ± 0.21

As can be observed in **Figure 6.1**, the evidently low RS concentration in the FPBR severely impacted the cumulative hydrogen production, reaching only 141 $mL \cdot L^{-1}$ at the end of the DF process, in contrast to the 1106 $mL \cdot L^{-1}$ observed in the RBR system. This behavior has been observed in previous studies reporting a positive correlation between sugar concentration in the system and cumulative hydrogen production (Nissilä et al., 2014; REN et al., 2006). Some authors observed that low sugar concentration may limit microbial growth, impacting the reaction efficiency. However, the available sugars are metabolized completely, yielding a higher H_2 volume per gram of substrate or volatile solids (Constante Fonseca et al., 2019; LI et al., 2008).

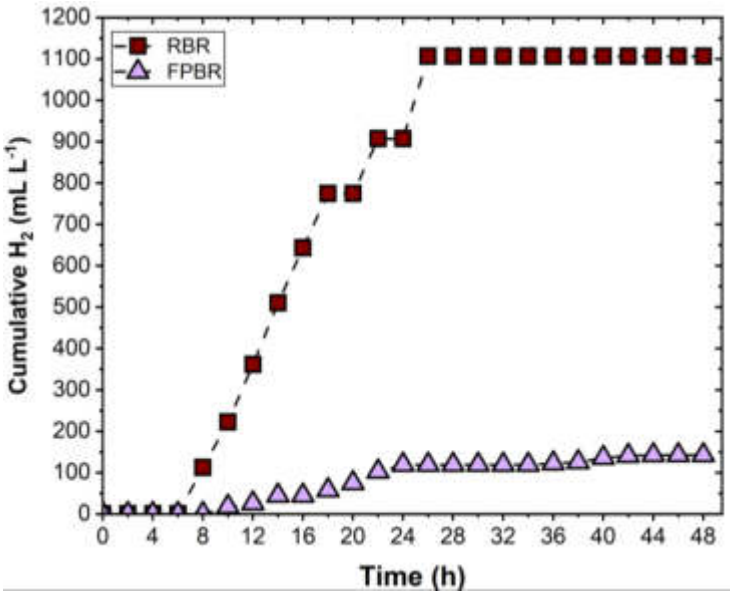


Figure 6.1. Cumulative H_2 production along the DF process in the batch reactors after 48 h (AS, 37°C, 2 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

The trend of lower H_2 accumulation when working with a lower initial RS concentration was also observed in the modified BHP test. However, in the latter experiment, the H_2 yield was higher than that obtained in the systems fed with untreated substrate, where the RS concentration was higher. A different trend was observed in the case of the 3 L batch reactors, where a substantially lower hydrogen yield was obtained in the FPBR compared to the RBR system (97 ± 10 vs 34 ± 2 $mLH_2 \cdot L^{-1}$), as shown in **Figure 6.2**. This discrepancy may be associated with the markedly lower RS concentration remaining in the fungal-pretreated substrate at larger scale operation. In the BHP tests, the increased accessibility generated by fungal pretreatment was sufficient to compensate for sugar losses through enhanced substrate conversion efficiency. However, under the operational conditions of the 3 L reactors, the severe depletion of readily fermentable sugars likely became the dominant limiting factor for hydrogen production. Additionally, the larger reactor volume may have increased the susceptibility of the process to substrate heterogeneity as well as mixing and mass-transfer limitations, phenomena that become more relevant during scale-up and may reduce the accessibility of the remaining fermentable sugars to the microbial community. Such scale-dependent

effects, which are generally negligible in small-scale BHP tests, may have further contributed to the lower hydrogen yield observed in the FPBR (Chezeau et al., 2019; Hallenbeck & Ghosh, 2009; Tenca et al., 2011). Overall, these results suggest that the balance between lignin degradation and carbohydrate preservation is critical when applying fungal pretreatments to lignocellulosic substrates for DF (Kainthola et al., 2021).

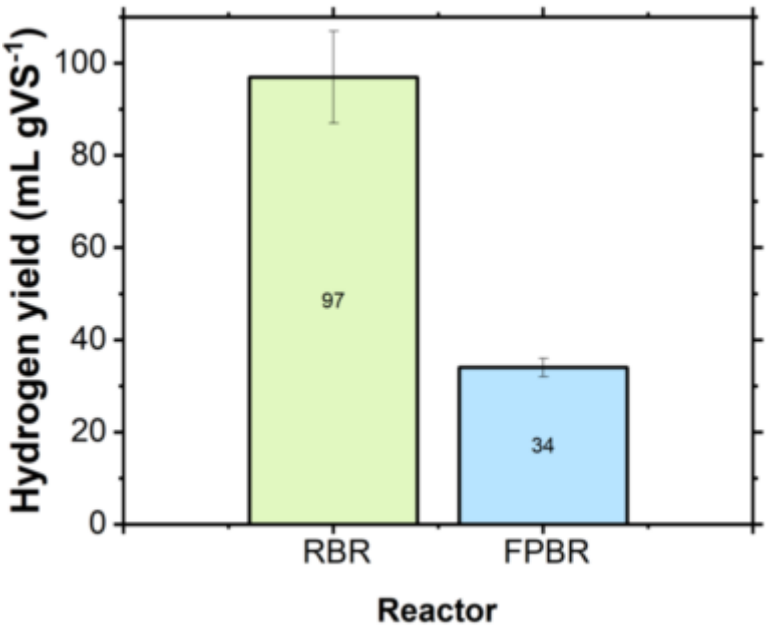


Figure 6.2. Comparative in H_2 yield observed in both batch reactors studied (AS, 37°C, 2 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

6.1.2. VFAs Accumulation and Microbial Community Analysis

With respect to the VFAs produced in the RBR and FPBR systems, these also differed from those observed in the previous BHP tests. Although, as shown in **Figure 6.3**, acetic and butyric acids remained the predominant metabolites, in this case, the RBR exhibited a slightly higher butyric acid concentration than the FPBR (0.10 vs 0.08 $g \cdot gVS^{-1}$), breaking the trend observed in the previous BHP assays, where the reactors fed with substrate pretreated with *T. versicolor* TV-38 in the BHP test and *T. versicolor* CIRM-BRFM 277 in the modified BHP test showed higher concentrations of acetic and butyric acids. Additionally, traces of propionic acid were detected in the RBR at the end of dark fermentation. However, despite its widely

known negative effect on hydrogen production, the RBR still exhibited superior performance in both cumulative hydrogen production and hydrogen yield compared to the FPBR (Sivagurunathan et al., 2014).

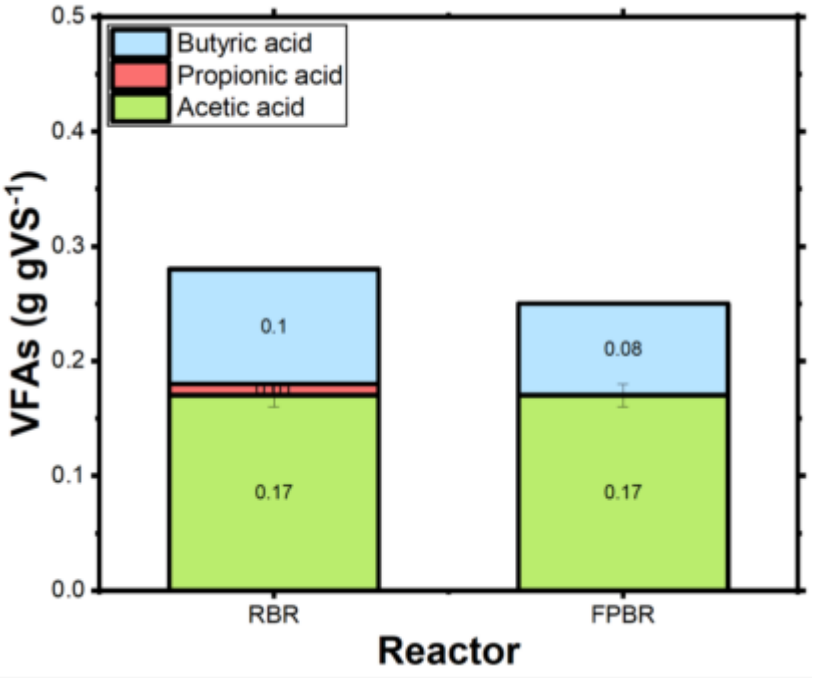


Figure 6.3. Cumulative VFA production after 48 h of fermentation in both batch reactors studied (AS, 37°C, WV 2 L, pH 5.50 ± 0.08, S/I 20, 150 rpm).

The maximum VFA accumulation was observed in the RBR system, reaching a total concentration of 0.280 g·gVS^{-1}, which, although higher than the final concentration observed in the FPBR, represented only a slight difference, as the latter reached a final concentration of 0.250 g·gVS^{-1}. Nevertheless, both concentrations were considerably lower than those observed in the modified BHP test fed with substrate pretreated with *T. versicolor*, where a final concentration of 0.430 g·gVS^{-1} was obtained. This phenomenon may be associated with pH control, since regulation of this parameter has been reported to influence VFA accumulation and distribution (Slezak et al., 2021). However, when comparing both systems fed with untreated substrate (RAW MIX in the modified BHP test and the RBR), a different VFA accumulation pattern was observed. The RBR system reached a total VFA concentration of 0.270 g·gVS^{-1}, whereas the RAW MIX evaluated in the modified

BHP test reached only 0.109 g·gVS^{-1}. Likewise, the RBR exhibited substantially higher hydrogen yield compared to the RAW MIX system (97 vs 42 mLH_2·gVS^{-1}), even when the HPE index was the same in both cases (0.63).

As previously mentioned, the FPBR system exhibited a total VFA concentration of 0.250 g·gVS^{-1} at the end of the process after 48 h, which was considerably lower than that observed in its counterpart evaluated in the modified BHP test, where a final concentration of 0.430 g·gVS^{-1} was reached in the SSF25d MIX system. This behavior broke the trend previously observed since the original BHP test, where higher final VFA concentrations were obtained when working with substrate pretreated with *T. versicolor*.

In terms of the HPE index, the FPBR exhibited a value of 0.22, which was lower than that observed in the SSF25d MIX system (0.28), indicating that part of the acetic acid produced was generated through the homoacetogenesis pathway, consuming hydrogen and negatively affecting its yield. As a result, the FPBR achieved a hydrogen yield of only 34 mLH_2·gVS^{-1}, compared to the 81 mLH_2·gVS^{-1} observed in the SSF25d MIX operated in the modified BHP test (Perat et al., 2024).

The considerable differences observed in hydrogen yield and VFA production in the batch systems compared to the BHP assays can be supported by the significant changes in the microbial communities present in the system. As shown in **Figure 6.4**, the microbial communities changed drastically with respect to those observed in the BHP tests, with the difference being more evident in the FPBR system. Previously, in the SSF25d MIX system, a clear predominance of the OTU associated with the genus *Clostridium* was observed (70%). However, in the FPBR, the relative abundance of the OTU related to *Clostridium* decreased to only 37%, while the OTU associated with *Enterobacter* became predominant, reaching 38 %, followed by *Klebsiella* with 8 %. On the other hand, in the RBR system, an evident predominance of the *Clostridium*-related OTU was identified, accounting for 77 % of the relative abundance and representing the most relevant microorganism identified in this reactor.

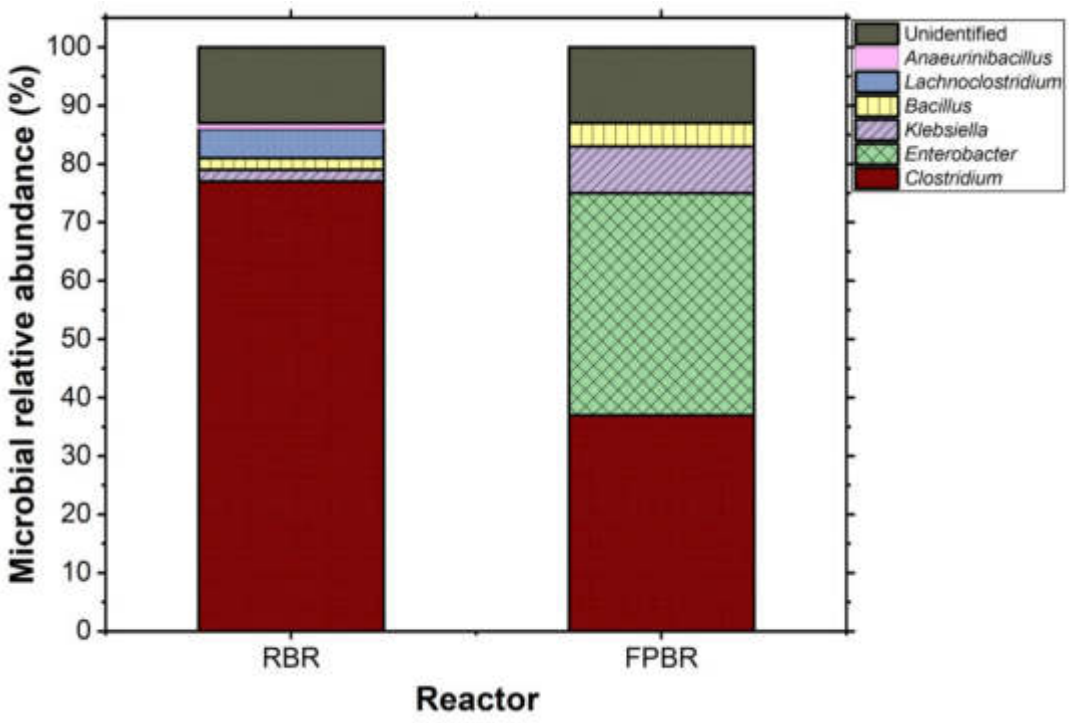


Figure 6.4. Microbial relative abundance at the end of DF in both batch reactors studied (AS, 37°C, 2 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

Although bacteria belonging to the genus *Enterobacter*, predominantly observed in the FPBR system, have been reported as hydrogen producers, these microorganisms are generally less efficient for this purpose compared to *Clostridium* species. In a study conducted by Torres de Souza et al., 2019, *Enterobacter* spp. were employed for hydrogen production, obtaining a yield of only 0.94 molH_2·mol glucose^{-1}. Additionally, unlike *Clostridium*, *Enterobacter* species are facultative anaerobic bacteria; therefore, their presence in the FPBR may be associated with residual oxygen in the system.

Despite supplying N_2 for 1 min in both batch reactors to establish anaerobic conditions at the beginning of the process, the purging time may have been insufficient for the FPBR. Since this reactor was considerably larger than the BHP systems, the efficiency of medium deoxygenation was likely lower than that achieved in the 200 mL reactors, which were subjected to the same purging time. This phenomenon may be reflected in the proliferation of *Enterobacter*, as facultative

anaerobic microorganisms commonly develop in systems containing residual oxygen.

Furthermore, the physicochemical nature of the substrate pretreated with *T. versicolor*, characterized by high porosity and the presence of fungal mycelium (Kainthola et al., 2021; Suryadi et al., 2022), may also have contributed to oxygen retention within the system. Lignocellulosic substrates colonized by fungal mycelia are capable of trapping gases, generating microspaces, and even forming internal oxygen gradients during pretreatment. Consequently, these conditions may have negatively affected the establishment of strict anaerobic conditions in the FPBR, impacting hydrogen yield, VFA production, and the selective proliferation of *Clostridium*, as previously observed in the modified BHP test (Hallenbeck & Ghosh, 2009; Oostra et al., 2001; Rouches et al., 2016b; Valdez-Vazquez & Poggi-Varaldo, 2009).

6.2. Setup of the 3 L Sequencing Batch Reactors

Once the results obtained from the batch reactors were analyzed, and in order to evaluate not only hydrogen production but also process stability and robustness, two different sequencing batch reactors were set up using, once again, raw substrate (RSBR) and fungal-pretreated substrate with *T. versicolor* CIRM-BRFM 277 (FPSBR). In this experimental stage, the same 3 L reactors previously used during the batch reactor experiments were employed, now operating at a working volume of 1.5 L under the same operational conditions established in the previous stage (37 °C, initial VS < 10 $g \cdot L^{-1}$, pH 5.50 ± 0.08, S/I 20, and 150 rpm).

The reactors were initially operated in batch mode and, once the stationary phase in hydrogen production was reached after 48 h, agitation was stopped for 20 min to allow biomass sedimentation within the system. After this settling period, 2/3 of the reactor working volume (1 L) was withdrawn and replaced with fresh substrate using a peristaltic pump, considering an initial VS concentration of <10 $g \cdot L^{-1}$. This procedure was repeated for a total of three feeding cycles.

Additionally, analyses of RS were performed at the beginning of the process, and VFAs as well as microbial communities at the end of each feeding cycle in order to evaluate the system evolution over time.

6.2.1. Hydrogen Production Performance and Physicochemical Analysis of the 3 L Sequencing-Batch Reactors

In this case, the DF process lasted up to 192 h after three feeding cycles. The first cycle lasted 48 h, the second 96 h, and the third 48 h. **Table 6.2** presents the initial physicochemical characteristics of both sequential batch reactors at each feeding cycle. Once again, lower concentrations of RS and solids were observed in the reactor fed with the substrate pretreated with Trametes versicolor (FPSBR).

Table 6.2. Initial physicochemical characteristics of each sequencing-batch reactor at the beginning of the three feeding cycles studied.

Reactor	Feeding cycle	RS (g·L-1)	TS (g·L-1)	VS (g·L-1)
RSBR	1	2.91 ± 0.07	9.74 ± 2.04	9.01 ± 2.01
FPSBR		0.42 ± 0.03	6.04 ± 0.65	4.93 ± 0.46
RSBR	2	2.43 ± 0.08	13.42 ± 0.37	11.44 ± 0.44
FPSBR		0.84 ± 0.23	12.50 ± 0.25	10.46 ± 0.30
RSBR	3	2.80 ± 0.05	12.83 ± 1.95	11.05 ± 1.70
FPSBR		1.12 ± 0.02	13.24 ± 0.16	11.10 ± 0.14

During the three feeding cycles, the initial RS concentrations ranged from 0.42 ± 0.03 to 1.12 ± 0.02 $g \cdot L^{-1}$ in the FPSBR, whereas the RSBR exhibited initial RS concentrations ranging from 2.80 ± 0.05 to 2.91 ± 0.07 $g \cdot L^{-1}$, representing more than twice the initial reducing sugar concentration observed in the FPSBR. A similar trend was observed for VS concentrations. The FPSBR presented initial VS concentrations ranging from 4.93 ± 0.46 to 11.10 ± 0.14 $g \cdot L^{-1}$, while the RSBR showed concentrations ranging from 9.01 ± 2.01 to 11.05 ± 1.70 $g \cdot L^{-1}$. Although the difference in VS concentrations was less pronounced than that observed for RS, a considerably larger disparity was detected during the first feeding cycle (9.01 ± 2.01 and 4.93 ± 0.46 $g \cdot L^{-1}$ for RSBR and FPSBR, respectively).

As observed in previous experiments, these differences can directly affect hydrogen accumulation within the system. The lower concentration of RS available in the FPSBR resulted in reduced cumulative H_2 production, owing to the

consumption of these substrates during the pretreatment stage (Constante Fonseca et al., 2019). This effect can be clearly observed in **Figure 6.5**, which illustrates the cumulative H_2 production throughout the three feeding cycles. The cumulative H_2 production obtained in the RSBR after 192 h of operation over the three feeding cycles was 4946 $mL \cdot L^{-1}$, representing a 9.5-fold increase compared with the cumulative H_2 production achieved in the FPSBR (520 $mL \cdot L^{-1}$).

These results once again revealed the evident inefficiency of the process when the substrate pretreated with *T. versicolor* was used, consistent with the observations previously reported for the batch reactors, in contrast with the BHP tests. However, despite achieving a cumulative hydrogen production of 4,946 $mLH_2 \cdot L^{-1}$ (26.03 $mLH_2 \cdot L^{-1} \cdot h^{-1}$) in the RSBR, this value remains lower than those reported in the literature for similar substrates.

For instance, (Buitrón & Carvajal, 2010) reported a hydrogen production rate of 50.5 $mLH_2 \cdot L^{-1} \cdot h^{-1}$ using tequila vinasse as substrate in a sequencing batch reactor operated at 35 °C and fed with 3 $gCOD \cdot L^{-1}$. Nevertheless, methane was detected in the biogas produced in that study, whereas in the present work, the only gaseous components identified were H_2 and CO_2.

On the other hand, an even higher H_2 production rate of 77.5 $mLH_2 \cdot L^{-1} \cdot h^{-1}$ was reported in a sequencing batch reactor fed with organic solid waste (Santiago et al., 2020). These findings suggest that, although hydrogen production was successfully achieved in the present study, further optimization of the operational conditions and substrate pretreatment strategy may be required to enhance the overall performance of the system.

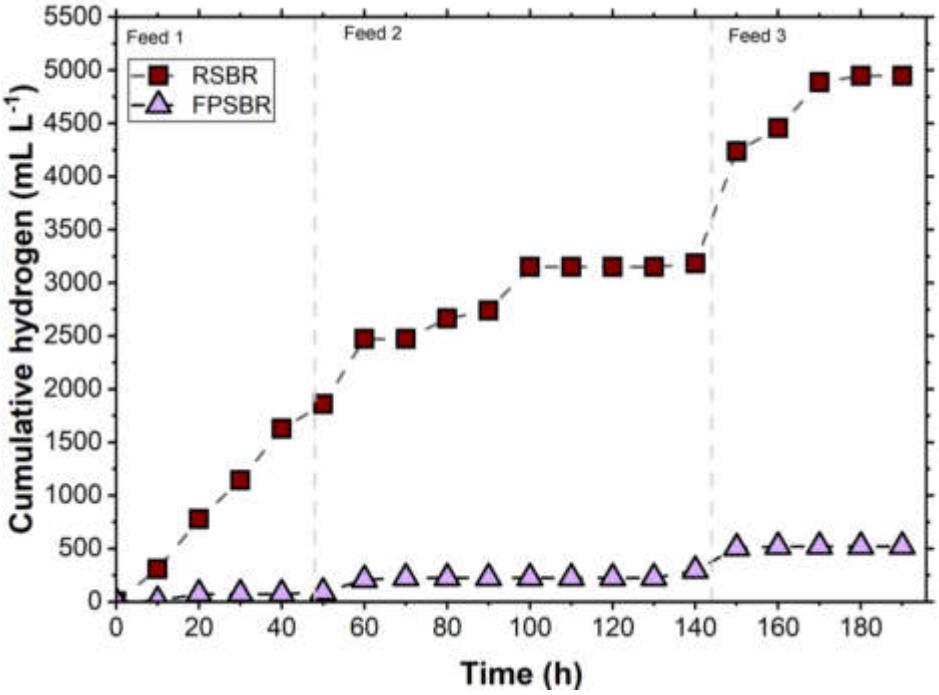


Figure 6.5. Cumulative H_2 production along the DF process in the sequencing batch reactors after 192 h (AS, 37°C, 1.5 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

Regarding the H_2 yield observed during the 192 h of reaction in both sequencing batch reactors, **Figure 6.6** displays the yield observed at the end of each feeding cycle. The same trend observed in the batch reactors was also identified in the sequencing batch reactors, where the untreated substrate exhibited superior hydrogen production performance. The RSBR achieved H_2 yields of 195 ± 44 $mLH_2 \cdot gVS^{-1}$ during the first feeding cycle, 164 ± 6 $mLH_2 \cdot gVS^{-1}$ during the second cycle, and 123 ± 19 $mLH_2 \cdot gVS^{-1}$ during the third cycle. In contrast, the FPSBR exhibited lower but relatively stable hydrogen yields throughout the sequencing batch operation, ranging from 18 to 20 $mLH_2 \cdot gVS^{-1}$.

Although the H_2 yield obtained in the RSBR was comparable to that observed in its batch-operated counterpart (RBR), the performance of the FPSBR declined relative to that achieved in the fungal-pretreated batch reactor (FPBR) during the previous experimental stage, decreasing from 34 ± 2 $mLH_2 \cdot gVS^{-1}$ to 20 ± 1 $mLH_2 \cdot gVS^{-1}$, corresponding to a reduction of approximately 41%.

This decrease may be associated with unfavorable microbial selection resulting from the limited availability of fermentable sugars in the medium, potentially favoring hydrogen-consuming microorganisms and promoting shifts in the metabolic pathways established within the FPSBR (Kim et al., 2005). Furthermore, the low concentration of reducing sugars may have negatively affected hydrogen production performance in the larger-capacity systems, such as the FPBR and FPSBR. Previous studies have reported that insufficient substrate availability can alter the distribution of fermentation products, reduce microbial activity, and ultimately impair hydrogen production during dark fermentation processes (Kim et al., 2005; Santiago et al., 2020).

Interestingly, this behavior was not observed in the previous BHP test, despite presenting lower concentrations of RS and VS than their counterparts fed with raw substrate, the fungal-pretreated systems achieved higher H_2 yields as well as greater accumulation of VFAs, particularly acetic and butyric acids. This phenomenon may be attributed to improved anaerobic conditions resulting from the N_2 flushing applied during the BHP tests. Although N_2 was supplied for 1 minute before DF, this procedure may have been more effective in the smaller BHP reactors than in the 3 L reactors used for batch and sequencing batch assays. Additionally, the smaller reactor volume may have promoted more efficient nutrient diffusion and reduced mass-transfer limitations or pH gradients, thereby enhancing microbial activity and hydrogen production performance when the fungal-pretreated substrate with a low sugar content was used (Arulrajah et al., 2025).

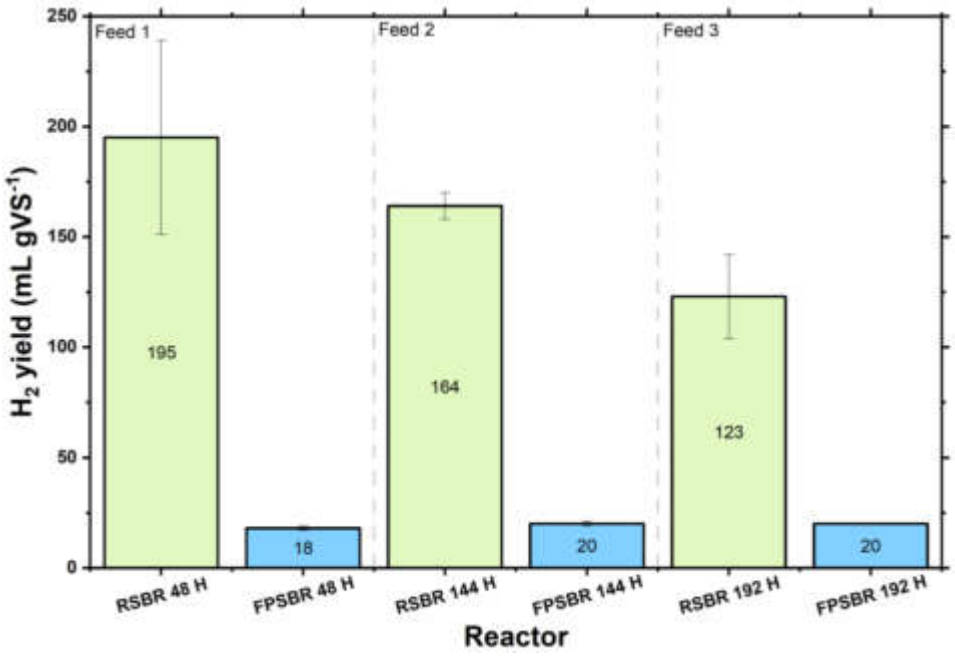


Figure 6.6. H_2 yield observed in each feeding cycle during the DF process in sequencing batch reactors (AS, 37°C, 1.5 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

6.2.2. VFAs Accumulation and Microbial Community Analysis

The trend of lower H_2 yields observed in the *T. versicolor*-pretreated system was also reflected in the production of VFAs throughout the process. As shown in **Figure 6.7**, VFA accumulation at the end of each feeding cycle was consistently lower in the FPSBR than in the RSBR. The total VFA accumulation in the RSBR at the end of each feeding cycle ranged from 0.31 to 0.35 $g \cdot gVS^{-1}$, whereas the total accumulation in the FPSBR ranged from 0.12 to 0.29 $g \cdot gVS^{-1}$.

Propionic acid was detected in the FPSBR at all sampling times, including the end of each feeding cycle and during an intermediate sampling taken in the second cycle (72 h). Its concentration ranged from 0.01 to 0.09 $g \cdot gVS^{-1}$. Although these concentrations were relatively low, the presence of propionic acid may have negatively affected hydrogen production and yield within the system, as propionic acid formation is generally considered an unfavorable metabolic pathway for hydrogen generation (Bernardo Ruggeri, Tonia Tommasi, 2015).

Furthermore, after 192 h of operation, trace concentrations of valeric acid and caproic acid were detected exclusively in the FPSBR, reaching 0.01 and 0.04 $g \cdot gVS^{-1}$, respectively. The presence of valeric acid, for instance, may indicate a diversion of metabolic fluxes away from hydrogen-producing pathways, since the accumulation of valeric acid has been associated with inhibitory effects on dark fermentative hydrogen production (Jones et al., 2015). On the other hand, caproic acid formation during DF is associated with chain elongation pathways involving ethanol, acetic acid, and butyric acid. This metabolic route can compete with hydrogen production because reducing equivalents are redirected toward caproic acid synthesis (Ding et al., 2010).

In contrast, no propionic acid was detected in the RSBR at the end of the first feeding cycle, with acetic acid (0.20 $g \cdot gVS^{-1}$) and butyric acid (0.13 $g \cdot gVS^{-1}$) being the only fermentation by-products identified. However, following the intermediate sampling performed during the second feeding cycle, propionic acid was detected at concentrations ranging from 0.01 to 0.09 $g \cdot gVS^{-1}$, suggesting a partial diversion of metabolic activity toward pathways that are less favorable for hydrogen production (Chatellard et al., 2016).

Nevertheless, acetic acid and butyric acid remained the predominant metabolites throughout the process in RSBR, reaching concentrations between 0.15 and 0.24 $g \cdot gVS^{-1}$ and between 0.09 and 0.13 $g \cdot gVS^{-1}$, respectively. These results further confirm the superior performance of the system fed with the untreated substrate, not only in terms of hydrogen yield but also with respect to VFA accumulation (Chatellard et al., 2016; Taheri et al., 2018). This behavior contrasts with the results obtained in the BHP tests conducted in the present study, where the fungal-pretreated substrate exhibited a more favorable performance.

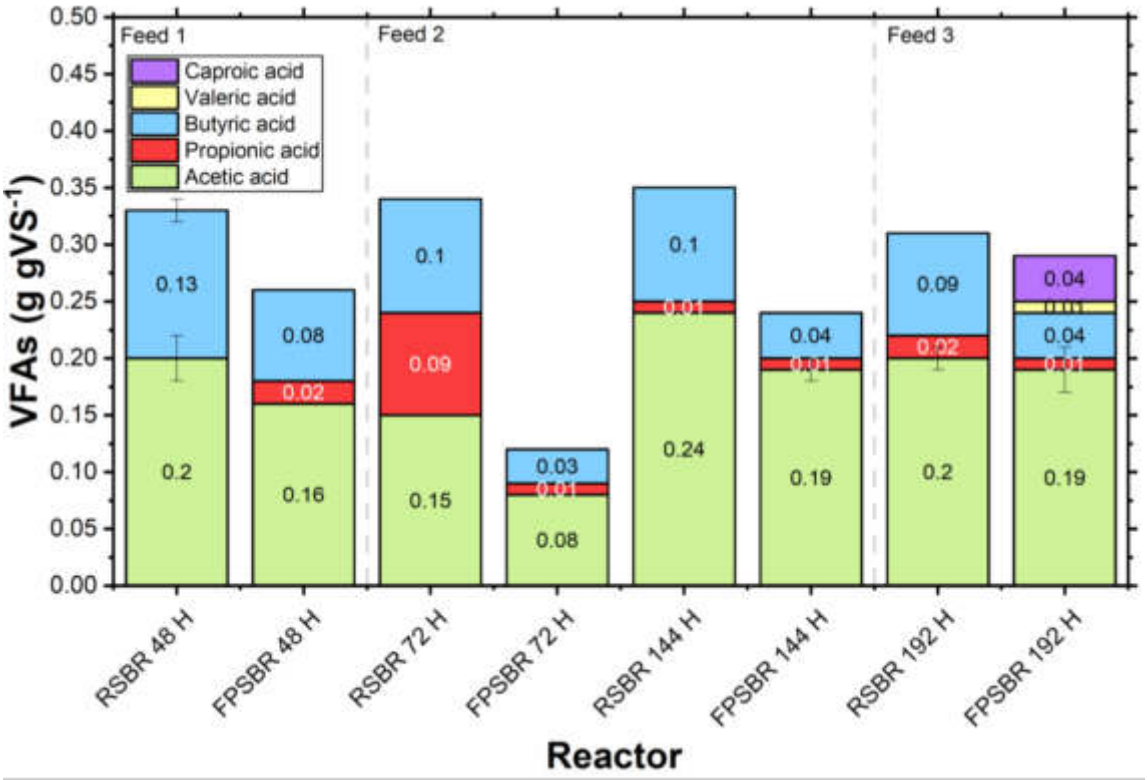


Figure 6.7. Cumulative VFA production at the end of each feeding cycle during 192 h of fermentation in both sequencing batch reactors studied (AS, 37°C, 1.5 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

Regarding microbial communities composition, a sample was taken at the end of each feeding cycle, as well as in the intermediate sample of the second feeding cycle. **Figure 6.8** shows the microbial relative abundance in each system at all sampling times. As can be observed, at the end of the first feeding cycle, after 48 h of operation, the relative abundance of the OTU associated with the genus *Clostridium* was considerably higher in the RSBR, accounting for 74% of the microbial community. In contrast, the FPSBR exhibited a relative abundance of only 34% for this OTU and was instead dominated by the facultative anaerobic genus *Enterobacter*, which represented 49% of the total community. This marked difference highlights the predominance of *Clostridium*, one of the most recognized hydrogen-producing bacterial genera, in the RSBR, where its relative abundance was more than double that observed in the FPSBR.

A similar pattern was observed in the batch reactors previously evaluated. The OTU associated with *Clostridium* represented 77% of the microbial community in the reactor fed with untreated substrate (RBR), whereas the fungal-pretreated batch reactor (FPBR) exhibited a relative abundance of only 37% for this OTU and 38% for the OTU associated with *Enterobacter*. These results, obtained in both batch and sequencing batch reactors after 48 h of operation, differ substantially from those observed in the BHP tests, where a clear predominance of hydrogen-producing bacteria (HPB) was detected in the systems fed with *T. versicolor*-pretreated substrate. This observation further supports the hypothesis that, in the larger-scale systems (batch and sequencing batch reactors), nitrogen sparging for 1 min was insufficient to ensure fully anaerobic conditions (Hallenbeck & Ghosh, 2009; Oostra et al., 2001; Rouches et al., 2016b; Valdez-Vazquez & Poggi-Varaldo, 2009).

During the second feeding cycle, both at the intermediate sampling point (72 h) and at the end of the cycle (144 h), this trend was reversed. The FPSBR exhibited a higher relative abundance of the OTU associated with Clostridium, reaching 64% and 62% at 72 and 144 h, respectively, whereas the corresponding values in the RSBR were 42% and 44%.

Nevertheless, at both 72 and 144 h of operation, the proliferation of additional bacterial genera was observed in both systems. In RSBR, *Enterococcus*, *Ruminococcus*, and *Caproiciproducens* were detected at 72 h with relative abundances of 10%, 6%, and 9%, respectively. By 144 h, the relative abundance of the OTU associated with the genus *Proteiniphilum* had increased to 19%. In contrast, the FPSBR exhibited a microbial community at 72 h that was largely similar to that observed at 48 h, with the main differences being an increase in the relative abundance of *Clostridium* (64%) and a decrease in the abundance of *Enterobacter* (15%). At the end of the second feeding cycle (144 h), the relative abundance of *Clostridium* remained relatively stable, and *Enterobacter* was no longer detected. However, the OTU associated with *Caproiciproducens* was identified at a relative abundance of 8%.

Finally, at the end of the third feeding cycle, after 192 h of operation, *Proteiniphilum* became the dominant genus in both systems, accounting for 51% and 37% of the microbial community in the RSBR and FPSBR, respectively. Simultaneously, the relative abundance of *Clostridium* decreased to 13% in the RSBR and 18% in the FPSBR. Additional OTUs associated with *Bifidobacterium* and *Caproiciproducens* were detected in the RSBR, with relative abundances of 14% and 11%, respectively. Similarly, *Caproiciproducens* was detected in the FPSBR at a relative abundance of 7%, together with the genus *Mobilitalea*, which accounted for 15% of the microbial community.

Although *Caproiciproducens* was detected in both systems, caproic acid was only identified as a fermentation by-product in the FPSBR after 192 h of operation (Bernardo Ruggeri, Tonia Tommasi, 2015; Ding et al., 2010). Furthermore, despite not being a hydrogen-producing bacterium, *Proteiniphilum*, the predominant OTU identified at the end of the process, is a hydrolytic and acidogenic anaerobe commonly associated with protein degradation into amino acids and the metabolism of organic acids (Feng et al., 2023). Its presence also suggests active conversion of complex organic matter into soluble intermediates and volatile fatty acids, which may indirectly enhance dark fermentative hydrogen production by supplying fermentable substrates for HPB (Feng et al., 2023; Moussa et al., 2022).

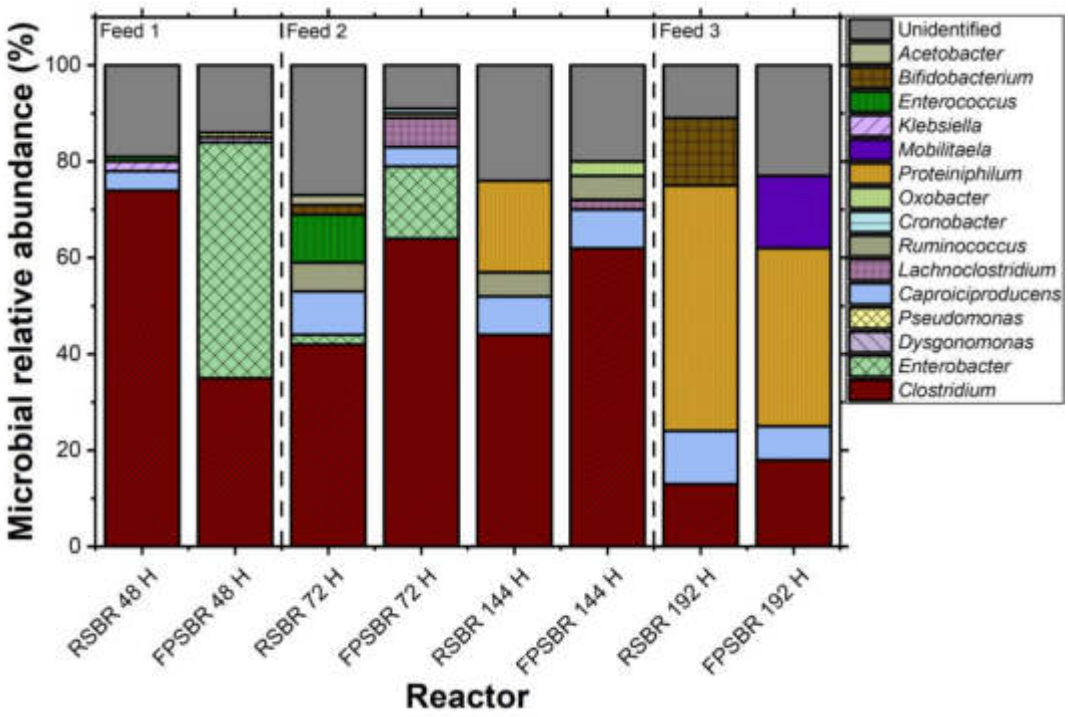


Figure 6.8. Microbial relative abundance at the end of each feeding cycle during 192 h of fermentation in both sequencing batch reactors studied (AS, 37°C, 1.5 L WV, pH 5.50 ± 0.08, S/I 20, 150 rpm).

CHAPTER 7

7. Conclusions

Based on the results obtained during the pretreatment stage, the BHP tests, and the batch and sequencing-batch reactor experiments, it can be concluded that hydrogen production from lignocellulosic residues pretreated with white-rot fungi using microbial consortia as inoculum is a viable approach. However, some considerations must be taken into account.

Fungal pretreatment resulted in effective delignification of the lignocellulosic mixture used as substrate. Based on the analysis of reducing sugar concentrations and Klason lignin content, the most favorable pretreatment condition among those evaluated was the use of *T. versicolor* at 28 °C for 25 days. In contrast, pretreatment with *P. ostreatus* led to a higher consumption of reducing sugars under all tested conditions during solid-state fermentation.

In the first BHP test conducted, an important improvement was observed in the TV-H group in terms of cumulative hydrogen production compared to the C-H group (341 ± 1 $mLH_2 \cdot L^{-1}$ vs. 166 ± 1 $mLH_2 \cdot L^{-1}$), hydrogen yield (19 $mLH_2 \cdot gVS^{-1}$ vs. 7 $mLH_2 \cdot gVS^{-1}$), and volatile fatty acids production at the end of the process (0.133 $g \cdot gVS^{-1}$ vs. 0.034 $g \cdot gVS^{-1}$). Additionally, the microbial community composition reflected an enrichment of HPB in the TV-H group, with relative abundances of 79% for the OTU affiliated with the genus *Klebsiella*, 13% for *Clostridium*, and 3% for *Escherichia*. These findings indicate that fungal pretreatment with *T. versicolor* for 25 days under solid-state fermentation conditions not only enhances dark fermentation performance in terms of hydrogen production and VFA accumulation but also alters the microbial community composition, directly influencing the metabolic pathways established within the system and promoting the accumulation of acetic and butyric acids.

The hydrogen yield was improved in the modified BHP test through the adjustment of specific operational conditions during dark fermentation, reaching 81 ± 9 $mLH_2 \cdot gVS^{-1}$ in the group fed with the *T. versicolor*-pretreated substrate (SSF25d MIX) and 42 ± 7 $mLH_2 \cdot gVS^{-1}$ in the control group fed with the untreated substrate (RAW MIX). These results once again demonstrated the beneficial effect of fungal

pretreatment on dark fermentation performance. A similar trend was observed for VFA production, with higher accumulation in SSF25d MIX (0.429 $g \cdot gVS^{-1}$) than in RAW MIX (0.109 $g \cdot gVS^{-1}$). Additionally, the improved buffering capacity of the pretreated-substrate systems was observed once again.

Regarding microbial relative abundance, the modified BHP test also showed an improvement compared to the original BHP test, with a higher relative abundance of the OTU associated with *Clostridium*. Furthermore, a positive effect of fungal pretreatment on the abundance of HPB was observed again, with OTUs related to the genera *Clostridium* (70%) and *Klebsiella* (9%) dominating the microbial community in SSF25d MIX. In contrast, RAW MIX was characterized by a relative abundance of 60% *Clostridium*, followed by 19% *Lactobacillus*, a bacterial genus generally associated with non-hydrogen-producing metabolic pathways. These findings further confirmed that fungal pretreatment promotes the enrichment of hydrogen-producing bacterial populations in dark fermentation systems, thereby enhancing both hydrogen yield and VFA accumulation.

Although fungal pretreatment with *T. versicolor* improved hydrogen production, VFA accumulation, and the enrichment of HPB in the BHP and modified BHP tests, these benefits were not maintained after scaling up the process to larger batch and sequencing-batch reactors. Under these operating conditions, reactors fed with untreated substrate achieved substantially higher hydrogen yields (97-195 $mLH_2 \cdot gVS^{-1}$) than those fed with fungal-pretreated substrate (20–34 $mLH_2 \cdot gVS^{-1}$). A similar trend was observed for VFA production, with untreated-substrate reactors exhibiting greater VFA accumulation. Microbial community analysis further revealed a higher abundance of HPB, particularly *Clostridium*, in the untreated systems, whereas fungal-pretreated reactors were characterized by a lower abundance of *Clostridium* and a greater relative abundance of *Enterobacter* after 48 h of reaction, for instance, which may have contributed to the reduced hydrogen yields observed.

A hypothesis emerging from the analysis of the experimental results is related to the effectiveness of oxygen removal during reactor start-up. In the batch and sequencing-batch reactors, a 1-min N_2 purge may not have been sufficient to

establish fully anaerobic conditions, in contrast to the smaller BHP test systems. This limitation may have particularly affected reactors fed with fungal-pretreated substrate, since the aerobic pretreatment performed by *T. versicolor* could have resulted in residual oxygen being retained within both the fungal mycelium and the substrate matrix. Furthermore, the increased porosity generated during fungal pretreatment may have facilitated oxygen retention within the biomass. This hypothesis is supported by microbial community analysis, which revealed the presence of OTUs affiliated with the genus *Enterobacter* after 48 h of operation in pretreated systems. Given that *Enterobacter* species are facultative anaerobes, their proliferation may represent a biological indicator of residual oxygen within the reactors.

The BMP test results indicated that fungal pretreatment with *T. versicolor* did not enhance substrate biodegradability, as similar biodegradability values were obtained for the pretreated and untreated substrates (20.99% vs. 23.80%, respectively). However, the improvements consistently observed in both BHP tests, including higher hydrogen yields, greater VFA accumulation, and a more favorable microbial community composition, suggest that fungal pretreatment primarily increased substrate bioavailability rather than biodegradability. Therefore, fungal pretreatment appears to have enhanced the accessibility of fermentable compounds to hydrogen-producing microorganisms, thereby improving dark fermentation performance despite having a limited effect on the overall substrate biodegradability.

7.1. Perspectives and Recommendations

Based on the results obtained from the four dark fermentation systems evaluated in this study (two BHP tests, batch reactors, and sequencing-batch reactors), it is recommended to carefully consider the initial concentration of reducing sugars, particularly in systems fed with fungal-pretreated substrates. Although reducing sugar concentration did not appear to negatively affect hydrogen yields in the BHP tests, it has been widely observed that this parameter directly influences the amount of biogas produced. Consequently, when very low concentrations of reducing sugars are available, both biogas production and hydrogen yield may be adversely affected.

Furthermore, additional pretreatment conditions should be investigated during solid-state fermentation to determine the optimal temperature, moisture content, and incubation time required to maximize lignin degradation while preserving fermentable reducing sugars. It would also be of great interest to evaluate other white-rot fungal (WRF) species that have been widely reported for their ligninolytic capabilities. These species could be assessed individually or in co-culture systems, with particular attention given to their enzymatic profiles and their effects on lignocellulosic biomass modification.

Likewise, fungal pretreatment could be applied to the effluents generated from dark fermentation systems fed with untreated substrates, with the aim of maximizing the utilization of lignocellulosic materials. Such an approach could enable the recirculation and further valorization of residual biomass, potentially leading to additional biogas and volatile fatty acid production while improving the overall efficiency of the process.

Another important research perspective concerns the optimization of overall process configuration. The integration of biological pretreatment, dark fermentation, and subsequent valorization of the fermentation residues should be evaluated under continuous or semicontinuous operation to determine the feasibility of long-term hydrogen production. Moreover, the possibility of integrating fungal pretreatment with other valorization pathways, such as the co-production of hydrogen and

methane or the recovery of bioactive compounds, could contribute to the development of a more sustainable biorefinery approach.

From an applied perspective, future studies should address the scalability and economic feasibility of the proposed strategy. Although fungal pretreatment represents an environmentally friendly alternative for improving lignocellulosic biomass accessibility, its implementation at industrial scale requires optimization of fungal cultivation time, energy requirements, substrate handling, and operational costs. Therefore, techno-economic analysis and life-cycle assessment studies are recommended to evaluate the environmental benefits and economic competitiveness of this integrated process compared with conventional waste management strategies.

Finally, the potential agricultural application of the residual biomass generated after fungal treatment and anaerobic fermentation should be further investigated. Considering the increasing interest in sustainable alternatives to synthetic fertilizers, the characterization of the resulting materials as biofertilizers or biostimulants could represent an additional valorization pathway. Future studies should evaluate their effects on plant growth, nutrient availability, soil microbial communities, and long-term agronomic performance, contributing to the transition toward circular bioeconomy models based on agroindustrial residues.

7.2. Dissemination

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White-rot fungi as a sustainable pretreatment approach to improve lignocellulosic biomass conversion for biohydrogen production

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ABSTRACT

The production of biofuels from lignocellulosic biomass is one of the most promising alternatives for generating clean energy. However, various challenges arise when using this recalcitrant biomass in biotechnological processes for the production of biofuels (ethanol, biogas, biohydrogen, and methane), such as low yields due to the structural complexity of lignocellulose. In this sense, this study evaluated a biological pretreatment of a lignocellulosic waste mixture consisting of fruit peels (FRP) and brewer's spent grain (BSG), through solid-state fermentation (SSF) using the white-rot fungi *Trametes versicolor* and *Pleurotus ostreatus*. Different pretreatment conditions were assessed, including duration (15, 25, and 35 days), temperature (28 and 32 °C), and fungal strain (*T. versicolor* and *P. ostreatus*), to determine their impact on reducing sugars concentration and delignification and improving the bioaccessibility of the biomass in further bioenergy obtention process.

The most suitable pretreatment condition was to carry out the SSF with *T. versicolor* at 28 °C for 25 days, obtaining a delignification of 15 ± 0.14 % and a reducing sugars consumption of 24 ± 0 %. The substrate pretreated under these conditions (TV-H) resulted in a 2.06-fold improvement in hydrogen production ($nLH_2\ L^{-1}$) compared to the raw substrate as well as a shift in microbial communities and metabolic pathways involved in comparison to the raw substrate in dark fermentation, demonstrating an improvement in the process when biomass is pretreated with *Trametes versicolor*.



The WHEC2024 Organizing Committee grants this

CERTIFICATE

Of recognition to:

Roberto Campos Flores, Nicolas Bernet, Santiago Suárez Vázquez, Eric Trably, Efrén Robledo Leal, Arquímedes Cruz López

For participating with the **Poster Presentation** entitlend:

" Impact of biological pretreatment of agri-industrial waste using white rot fungi prior to hydrogen production through dark fermentation " during

24th World Hydrogen Energy Conference 2024

held in Tulúm-Rivera Maya, México

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la Asociación de Biotecnología, Ingeniería Ambiental y Energías Renovables, Asociación Civil (ABIAER, AC)

Otorgan la presente constancia de participación a:

Campos-Flores, R. C.; Bernet, N., Suarez-Vázquez, S. I.; Trably, E.; Robledo-Leal, E. R.; Cruz-López, A.

Por la **PRESENTACIÓN ORAL** del trabajo titulado:

"Producción de bioH_2 a través de fermentación oscura utilizando residuos lignocelulósicos pretratados con hongos de pudrición blanca"

Durante el Simposio Ambiente y Bioenergía 2024 desarrollado los días 12, 13 y 14 de noviembre del 2024 en Boca del Río, Veracruz, México.

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29/07/26

SPEAKER CERTIFICATE

This is to certify that

Roberto CAMPOS

presented the **Oral standard** contribution entitled :

Number **281** – **Effect of white-rot fungal pretreatment on hydrogen yield by coupling solid-state fermentation and dark fermentation**

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at the 11th International Conference on Engineering for Waste and Biomass Valorisation held in A Coruña from July 6 to July 10, 2026.

Yours sincerely,

Prof Ange Nzihou
WasteEng2026 Chair

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APPENDIX

Detailed Experimental Methodology

- **Chemical Oxygen Demand (COD)**

COD represents the total amount of organic matter present in an aqueous sample. This parameter was determined using the Lovibond COD Vario Tube Test (0-1500 $mg \cdot L^{-1}$). The method is based on the oxidation of organic matter by potassium dichromate in a strongly acidic medium under high temperature conditions. During digestion, oxidizable compounds present in the sample reduce dichromate ions, and the resulting change in color intensity is measured photometrically and correlated with COD concentration.

A volume of 2 mL of sample was taken into the digestion tube containing the pre-dosed reagents. A reagent blank was prepared using distilled water following the same procedure.

Each tube containing a sample was capped, mixed, and placed in a COD reactor preheated to 150°C. Samples were digested for 120 minutes and subsequently allowed to cool to room temperature for 20 minutes. After cooling, the tubes were inverted three times to ensure homogenization of the reaction mixture.

Subsequently, COD concentration was determined using a uv-vis spectrophotometer Aqualytic MultiDirect with a wavelength of 600 nm, previously calibrated for the COD Vario Tube Test Medium Range (0-1500 $mg \cdot L^{-1}$). The instrument automatically converted absorbance values into COD concentrations expressed as $mgO_2 \cdot L^{-1}$. Samples exceeding the upper detection limit were appropriately diluted with distilled water and reanalyzed. All analyses were performed in duplicate, and the average value was reported.

- **Klason lignin**

Klason lignin content was determined using the standard two-step acid hydrolysis method. This procedure is based on the hydrolysis and solubilization of structural carbohydrates using concentrated and diluted sulfuric acid, while the acid-insoluble lignin fraction remains as a solid residue. The remaining residue is recovered by filtration, dried to constant weight, and quantified gravimetrically as Klason lignin, following this procedure:

1. 0.5 g of sample was weighed in an Erlenmeyer flask.
2. 7.5 mL of H_2SO_4 72% was added gradually under continuous stirring while maintaining the Erlenmeyer flask in a cold-water bath at 2 ± 1 °C.
3. Once the lignocellulosic substrate was completely dispersed, the flask was covered with a watch glass and maintained in a water bath at 20 ± 1 °C for 2 h, stirring frequently.
4. After 2 h hydrolysis period, the sample was transferred to a 500 mL Erlenmeyer flask, and 180 mL of distilled water was added to dilute the sulfuric acid concentration to approximately 3%.
5. The suspension was heated without boiling for 4 h while maintaining a constant volume with distilled water.
6. The suspension was allowed to settle and recover the solid fraction by vacuum filtration using Gooch crucibles fitted with Whatman filters (Cat. No. 1827-021) connected to Kitasato flasks.
7. The Gooch crucibles containing the filter and retained residue were dried at 105 °C for 24 h and weighed periodically until a constant weight was achieved.
8. Klason lignin content (%) was calculated by dividing the dry weight of the acid-insoluble residue (Klason lignin) by the initial weight of the substrate and multiplying the result by 100.

- **pH**

For solid samples, such as those analyzed during substrate characterization, pH was determined using a 5% (w/v) suspension of the corresponding sample in distilled water. After preparation, the suspension was allowed to settle for 20 min prior to pH measurement. Measurements were performed in triplicate using a pH electrode until consecutive readings differed by no more than 0.02 pH units. The mean pH value and corresponding standard deviation were subsequently recorded.

- **Reducing Sugars (RS) by DNS method**

The DNS (3,5-dinitrosalicylic acid) method is based on a colorimetric reaction for the quantification of reducing sugars. The DNS reagent is initially yellow; however, upon reaction with reducing sugars, it develops a dark brown color whose intensity is proportional to the concentration of reducing sugars present in the sample.

The DNS reagent was prepared by dissolving 2.5 g of 3,5-dinitrosalicylic acid and 75 g of sodium potassium tartrate in 200 mL of 0.5 N NaOH. The mixture was heated and continuously stirred until complete dissolution of the reagents. The final volume was then adjusted to 250 mL with distilled water.

Reducing sugars were determined according to the following procedure:

1. 1 mL of DNS reagent was transferred into a test tube.
2. 1 mL of sample or blank solution was added.
3. The sample was heated to boiling and allowed the reaction to proceed for 5 min.
4. The tubes were cooled in an ice bath for 5 min.
5. The final volume was adjusted to 10 mL by adding 8 mL of distilled water and was mixed vigorously using a vortex.
6. The absorbance was measured at 540 nm using a UV-Vis spectrophotometer.

- **Total carbohydrates (TCH) by the Dubois method**

Total carbohydrate content was determined using the phenol–sulfuric acid method. This colorimetric assay is based on the dehydration of carbohydrates by concentrated sulfuric acid to form furfural and hydroxymethylfurfural derivatives, which subsequently react with phenol to produce a colored complex. The intensity of the resulting color is proportional to the total carbohydrate concentration. To determine this parameter, the following procedure was carried out:

1. 1 mL of sample or blank solution was transferred into a test tube.
2. 0.5 mL of previously prepared 5% phenol solution and 3.5 mL of concentrated H_2SO_4 were added to the test tubes.
3. The samples were mixed vigorously using a vortex mixer for 10 s.
4. The tubes were allowed to cool for 10 min at room temperature.
5. The absorbance was measured at 490 nm using a UV-Vis spectrophotometer.

- **Total Nitrogen (TN) – Method 1 standardized Hach method (Hach kit 10-150 mg·L^{-1})**

This procedure was performed to determine the total nitrogen (TN) content of the substrates used in the preparation of the first lignocellulosic mixture. For this purpose, HACH Method 10072 was followed, which is based on a persulfate digestion using the manufacturer's TN determination kit with a concentration range of 10–150 mg·L^{-1}. The procedure was carried out as follows:

1. The digestion reactor was preheated to 105 °C.
2. The persulfate reagent packet supplied with the kit was added to the digestion tube, followed by the addition of 0.5 mL of sample.
3. The tubes were capped and vigorously shaken for 30 s.
4. Once the digestion reactor reached 105 °C, the tubes were placed inside and allowed to digest for 30 min.
5. After the digestion period was completed, the tubes were removed from the reactor and allowed to cool at room temperature for 10 min.

6. Reagent A from the TN kit was added to each tube, which were then vigorously shaken for 30 s and allowed to react for 3 min.
7. After the 3 min reaction, Reagent B from the TN kit was added. The tubes were capped and vigorously shaken for 15 s. The solution developed a yellowish color and was allowed to react for an additional 2 min.
8. A 2 mL aliquot was withdrawn from the digestion tube containing Reagents A and B and transferred to the TN kit tubes containing Reagent C. The tubes were inverted ten times and allowed to react for 5 min.
9. After the reaction period, the absorbance was measured using a UV–Vis spectrophotometer at a wavelength of 400 nm.

- **Total Nitrogen (TN) – Method 2 standardized Hach method LCK338 kit (20–100 mg·L^{-1})**

TN of the second lignocellulosic mixture was determined using the Hach LCK338 test (20-100 mgTN·L^{-1}), which is based on alkaline persulfate digestion followed by spectrophotometric determination of nitrate formed during digestion.

1. A volume of 0.2 mL of sample was transferred into the digestion cuvette provided in the kit.
2. Subsequently, 2.3 mL of Solution A and one oxidizing reagent tablet (Solution B) were added.
3. The cuvettes were tightly capped and digested at 120°C for 30 min in a Hach heating block.
4. After digestion, the cuvettes were allowed to cool to room temperature.
5. A volume of 0.5 mL of the digested sample was transferred to the analysis cuvette.
6. Then, 0.2 mL of Solution D was added, and the mixture was homogenized.
7. The reaction mixture was allowed to stand for 15 min at room temperature for color development.
8. Absorbance was measured using a Hach DR3900 spectrophotometer, and TN concentration was expressed as mgTN·L^{-1} according to the manufacturer's calibration.