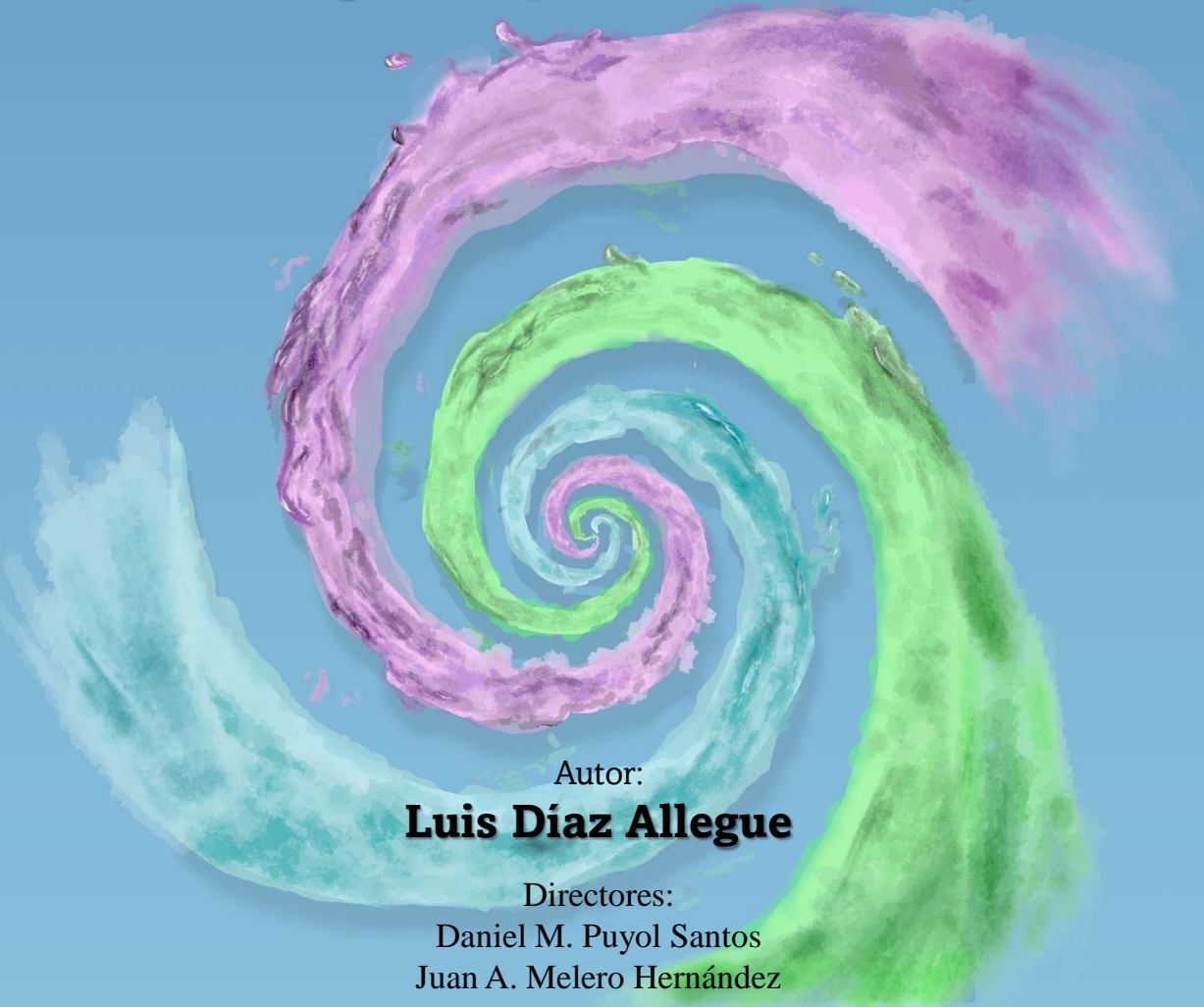# Universidad Rey Juan Carlos

TESIS DOCTORAL

Valorisation of the organic fraction of municipal solid waste waste via an integrated photobiorefinery

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Escuela Internacional de Doctorado

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Programa de Doctorado en Tecnologías Industriales: Química, Ambiental, Energética, Electrónica, Mecánica y de los Materiales

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Madrid, 2022

D. Juan Antonio Melero, Catedrático de Universidad en el área de Ingeniería Química del Departamento de Tecnología Química y Ambiental de la Universidad Rey Juan Carlos,

y

D. Daniel Melchor Puyol Santos, investigador Ramón y Cajal en el área de Ingeniería Química del Departamento de Tecnología Química y Ambiental de la Universidad Rey Juan Carlos,

Certifican:

Que el presente trabajo de investigación, titulado: "*Valorisation of the organic fraction of municipal solid waste via an integrated photobiorefinery*" constituye la memoria que presenta **D. Luis Diaz Allegue** para aspirar al grado de Doctor en Ingeniería Química por la Universidad Rey Juan Carlos, y que ha sido realizado en los laboratorios del Grupo de Ingeniería Química y Ambiental de la Universidad Rey Juan Carlos bajo nuestra supervisión.

"To be truly radical is to make hope possible,

rather than despair convincing"

Raymond Williams

RESUMEN

La presente Tesis Doctoral ha sido realizada en el Departamento de Tecnología Química y Ambiental de la Universidad Rey Juan Carlos, englobada en la línea de investigación de "Valorización y caracterización de materiales residuales en el marco de la economía circular", siendo financiada principalmente por el proyecto BIOTRES (S2018/EMT-4344 BIOTRES-CM, https://madrid.bio3project.es/) de la Comunidad de Madrid. Asimismo, se ha realizado una estancia predoctoral en el Instituto de Investigación en Biociencias de la Universitè de Mons del 3 de mayo al 3 de agosto de 2021 durante la rea lización de la Tesis Doctoral.

La transición desde la actual economía lineal a una economía circular es uno de los grandes retos de nuestra sociedad. Uno de los principales motores de este cambio de paradigma es el previsible agotamiento de los recursos materiales. La gestión eficiente de los recursos se convierte, por tanto, en algo esencial. El incremento de la generación de residuos sólidos municipales supone un coste medioambiental y económico para la sociedad, pero a la vez una fuente de recursos que no puede ser desaprovechada. Más de la mitad de estos residuos es material biodegradable, conocido como la fracción orgánica de los residuos solidos municipales (FORSU) y que constituye una fuente abundante de carbono.

La necesidad de garantizar el suministro de materiales y energía, así como de minimizar la dependencia de los combustibles fósiles, ha impulsado el concepto de las biorrefinerías, que es análogo al de las refinerías basadas en el petróleo, pero que utiliza residuos orgánicos en lugar de petróleo como materia prima. El principal objetivo de una biorrefinería es maximizar el valor derivado de los componentes y productos intermedios convirtiéndolos en una gama de valiosos bioproductos y bioenergía. Uno de estos productos de alto valor añadido son los bioplásticos, candidatos a substituir los plásticos derivados del petróleo, omnipresentes en nuestra vida y que causan serios problemas medioambientales y a los ecosistemas. Dentro de los posibles sustitutos, los polihidroxialcanoatos (PHA) son una alternativa con propiedades químicas, térmicas y mecánicas similares a los plásticos derivados del petróleo,

pero más amigables con el medio ambiente, ya que son biodegradables y compostables, por lo que son un componente perfecto para entrar en las cadenas de producción de una economía circular.

Actualmente, la producción de PHA no ha conseguido ser económicamente competitiva frente a los plásticos tradicionales. La producción industrial de PHA actualmente consiste en procesos con cultivos puros y materias primas simples como azúcares, lo cual encarece la producción debido a los altos costes de esterilización y de esta materia prima. Para reducir costes, en los últimos años se está estudiando procesos con cultivos mixtos, que no requieren esterilización, y la utilización de residuos como materia prima. Para reducir aún más los costes, el uso de bacterias fototróficas purpura (BFP) constituye un prometedor mecanismo para la industria debido a su alta versatilidad metabólica y a que no necesitan oxígeno para la acumulación de PHA.

El metabolismo más común de las BFP es el fotoheterotrófico, con el cual son capaces de usar los fotones emitidos por el sol como fuente de energía y compuestos orgánicos como fuente de carbono y electrones. Este perfil metabólico optimiza el uso de los compuestos orgánicos, ya que no deben derivar una parte para la producción de energía, como sí tienen que hacer los cultivos quimiotróficos, pudiéndose obtener así mayores rendimientos de recuperación de carbono. Es por ello que cultivos mixtos de BFP pueden reducir los costes de producción de PHA y abrir nuevas vías biotecnológicas, ya que pueden usar ácidos carboxílicos de cadena corta, derivados de procesos de fermentación, como substratos baratos y fácilmente disponibles y, además, utilizar el sol como fuente de energía.

Nunca antes se había intentado llevar a cabo la valorización completa de la FORSU utilizando como tecnología central los cultivos mixtos de BFP para producir productos de alto valor añadido. La mayoría de los trabajos realizados con cultivos mixtos de BFP se centran en el tratamiento de aguas residuales, y los que tratan sobre acumulación de PHA emplean casi exclusivamente cultivos puros. El estudio de una fotobiorefiniera integrada, donde se concatenen pretratamientos térmicos y procesos biotecnológicos para valorizar la FORSU y conseguir cerrar los ciclos de carbono y energía de manera competitiva representa un reto aun por explotar. Además, otra pregunta sin respuesta son las diferentes rutas de acumulación de carbono de los cultivos mixtos de BFP según diferentes estados redox y de estrés.

Como la FORSU es extremadamente heterogénea y puede contener compuestos tóxicos, el primer reto de esta tesis es comprobar que un pretratamiento de hidrolisis térmica puede crear una corriente liquida con la cual las BFP puedan crecer y acumular PHA. En a **Sección I** se propone el estudio de una prueba de concepto de biorrefinería donde se encadena el pretratamiento por hidrolisis térmica con una digestión anaerobia de la fracción solida resultante y un proceso fotoheterotrófico con BFP de la fracción liquida. Esta prueba de concepto se estudió con FORSU real de recogida selectiva y preclasificada en una planta de tratamiento, así como en residuo lignocelulósico de poda de parques. Los resultados mostraron una solubilización de materia orgánica (DQO) en la hidrólisis térmica de hasta un 40%, con ratios muy altos de DQO/nutrientes, siendo más importante la temperatura que el tiempo de reacción. Además, el pretratamiento mejora la biodegradabilidad general de la FORSU, compensando en su potencial metanogénico la perdida de parte del carbono de la fracción liquida. El biogás producido podría alimentar una planta de cogeneración (PCG), con resultados de generación de energía térmica y eléctrica que podrían hacer el proceso energéticamente autosostenible. En cuanto a la asimilación de la corriente líquida por el cultivo mixto de BFP, se consigue un consumo de hasta el 80% de la DQO con rendimientos de biomasa de hasta 0,5 gSSV gDQO^{-1}. Además, se alcanzaron acumulaciones de hasta el 21% en materia seca de PHA, confirmando la viabilidad de la prueba de concepto propuesta.

Para mejorar los rendimientos de biomasa y de producción de PHA, se conoce que las BFP asimilan con mayor facilidad compuestos orgánicos reducidos como los ácidos carboxílicos de cadena corta (acido acético, propiónico, butírico, etc.). Por ello, se propone un paso de pretratamiento intermedio, la fermentación acidogénica. En la **Sección II** se estudia la viabilidad de la co-fermentación a temperaturas termofílicas de los dos componentes más abundantes de la FORSU: residuos de comida y residuo lignocelulósico. Esta fermentación se estudia sin y con pretratamiento térmico mediante explosión de vapor. El pretratamiento con explosión de vapor mejora considerablemente la solubilización de materia orgánica y, tras ambos tratamientos, se consiguen porcentajes de solubilización de hasta el 80% de la DQO en la corriente líquida. Además, la fermentación acidogénica produce simultáneamente H_2, consiguiéndose en estos ensayos producciones de hasta 162 ± 5 mlH_2 gDQO^{-1}. Los resultados mostraron claramente un efecto sinérgico positivo en la eficiencia global de la co-fermentación entre ambos residuos y

una fuerte dependencia en la distribución de ácidos carboxílicos. Se alcanzaron valores de hasta 0,58 gDQO de ácidos carboxílicos por gDQO añadido con el pretratamiento térmico, y una acidificación del 93% (%DQO referido a ácidos carboxílicos).

Habiendo demostrado que la combinación de pretratamiento térmico y fermentación acidogénica termofílica permite obtener una corriente liquida con alta carga en ácidos carboxílicos de cadena corta, en la **Sección III** se procedió a estudiar la fotobiorefineria propuesta (Figura R1). Se utiliza un fotobiorreactor de membrana (FBRM) para tratar el efluente liquido de un fermentador acidogénico en continuo alimentado con hidrolizado de FORSU tras steam explosion. La fracción solida obtenida tras la fermentación la valorizamos mediante digestión anaerobia.
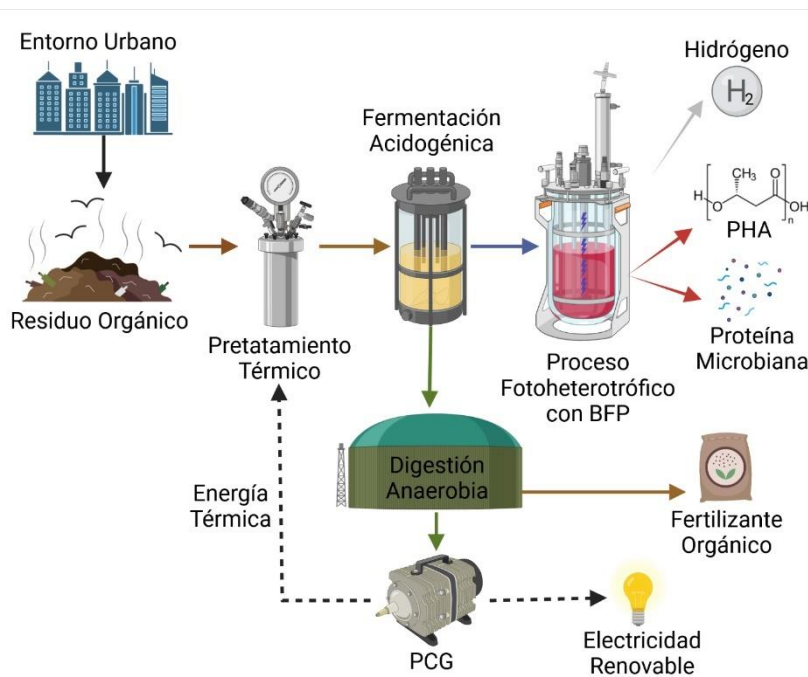
Figura R1. Representación esquemática de la fotobiorrefinería de tratamiento de residuos propuesta.

En este estudio se intenta optimizar el crecimiento de biomasa y la acumulación de PHA en el FBRM ajustando la carga orgánica que reciben las BFP. Se alcanzó una acumulación máxima de PHA del 42% en materia seca, el máximo obtenido hasta ahora en un cultivo mixto de BFP alimentado con residuos. Se han detectado PHA de cadena media como el

polihidroxihexanoato, con mejores propiedades físico-químicas en comparación con los PHA de cadena corta, que podrían diversificar sus aplicaciones industriales. Se muestran también por primera vez alternativas a la acumulación de PHA: el almacenamiento de carbono tanto en el glucógeno como en los polímeros extracelulares (PEC), al tiempo que se deriva el exceso de electrones en H_2 incluso en presencia de amonio orgánico. Además, se ha realizado un estudio estadístico de la evolución de las comunidades bacterianas para determinar la influencia de las variables ambientales sobre su variabilidad, como por ejemplo la variación de géneros en el FBRM tras un shock de carga orgánica o que géneros son los mas prominentes cuando se acumula PHA. Por último, se realizaron balances de materia y energía preliminares que indican que la fotobiorefinería integrada propuesta puede ser energéticamente sostenible, a la vez que produce 109 kg de PHA por tonelada de sólidos totales de FORSU. Estos resultados resultan prometedores para una implementación a mayor escala de una fotobiorrefinería basada en BFP.

Por último, en la **Sección IV** se muestra una revisión critica sobre el escalado del proceso más innovador de la fotobiorefinería propuesta, el proceso foheterotrófico con BFP. El éxito y la viabilidad económica de la producción a escala industrial de biopolímeros depende principalmente de cómo enriquecer cultivos mixtos de BFP capaces de acumular grandes cantidades de PHA y los principales cuellos de botella para el escalado de esta tecnología. Se discuten las ventajas y desventajas de las estrategias de acumulación de PHA, como la de festín y hambruna o festín permanente, teniendo esta última la ventaja clara de poder recuperar hasta el 100% de carbono (acercando el sistema a la neutralidad de CO_2) con una alta conversión en PHA. En cuanto al escalado se concluye que, posiblemente, la mejor opción sea reactores al aire libre, principalmente utilizando la iluminación solar natural como fuente de energía. Sin embargo, se indican muchas estrategias que pueden aumentar el rendimiento del proceso, como sistemas de captación y filtración solar y dilución espacial de la luz, así como estrategias de floculación, granulación, homogenización y automatización del sistema que pueden resultar útiles para la reducción de costes, pero que deben ser investigadas en mayor profundidad.

En conjunto, este trabajo ha propuesto y probado una fotobiorefineria a TRL < 3 con el objetivo de valorizar FORSU en PHA y otros productos de alto valor añadido como el biogás, el H_2 o la proteína microbiana. Además, los resultados de este trabajo ayudan a entender mejor el proceso

fotoheterotrófico de producción de PHA mediante BFP con el objetivo de preparar el camino para su escalado industrial.

RESUMO

Esta Tese de Doutoramento realizouse no Departamento de Tecnoloxía Química e Ambiental da Universidade Rey Juan Carlos, incluída na liña de investigación "Valorización e caracterización de materiais residuais no marco da economía circular", sendo financiada principalmente pola BIOTRES. proxecto (S2018/EMT-4344 BIOTRES-CM, https://madrid.bio3project.es/) da Comunidade de Madrid. Así mesmo, realizouse unha estancia predoutoral no Instituto de Investigación en Biociencias da Universitè de Mons do 3 de maio ao 3 de agosto de 2021 durante a realización da Tese Doutoral.

O paso da economía lineal actual a unha economía circular é un dos grandes retos da nosa sociedade. Un dos principais motores deste cambio de paradigma é o previsible esgotamento dos recursos materiais. A xestión eficiente dos recursos faise, pois, imprescindible. O aumento da xeración de residuos sólidos urbanos supón un custo ambiental e económico para a sociedade, pero á vez tempo unha fonte de recursos que non se pode desperdiciar. Máis da metade destes residuos son material biodegradable, coñecido como a fracción orgánica dos residuos sólidos urbanos (FORSU) que constitúe unha fonte abundante de carbono.

A necesidade de garantir o abastecemento de materiais e enerxía, así como de minimizar a dependencia dos combustibles fósiles, impulsou o concepto de biorrefinerías, que é análogo ao das refinerías de petróleo, pero que empregan no seu lugar biomasa ou residuos. O obxectivo principal dunha biorrefinería é maximizar o valor derivado dos compoñentes e produtos intermedios converténdoos nunha gama de bioprodutos e bioenerxía valiosos. Un destes produtos de alto valor engadido son os bioplásticos, candidatos a substituír os plásticos derivados do petróleo, omnipresentes nas nosas vidas e que causan graves problemas ambientais e dos ecosistemas. Entre os posibles substitutos, os polihidroxialcanoatos (PHA) son unha alternativa con propiedades químicas, térmicas e mecánicas semellantes aos plásticos derivados do petróleo, pero máis amigables co medio ambiente, xa que son biodegradables e compostables, polo que son un compoñente perfecto para entrar nas cadeas de produción dunha economía circular.

Actualmente, a produción de PHA non conseguiu ser economicamente competitiva fronte aos plásticos tradicionais. A produción industrial de PHA consiste na actualidade en procesos con cultivos puros e materias primas sinxelas como os azúcres, o que encarece a produción polos altos custos de esterilización e desta materia prima. Para reducir custos, nos últimos anos estudáronse procesos con cultivos mixtos, que non precisan de esterilización, e a utilización de residuos como materia prima. Para reducir aínda máis os custos, o uso de bacterias fototróficas vermellas (BFV) constitúe un mecanismo prometedor para a industria debido á súa alta versatilidade metabólica e ao feito de que non necesitan osíxeno para a acumulación de PHA.

O metabolismo máis común das BFV é o fotoheterótrofo, co que son capaces de utilizar os fotóns emitidos polo sol como fonte de enerxía e os compostos orgánicos como fonte de carbono e electróns. Este perfil metabólico optimiza o uso de compostos orgánicos, xa que non teñen que derivar unha parte para a produción de enerxía, como teñen que facer os cultivos quimiotróficos, podendo así obter maiores rendementos de recuperación de carbono. É por isto que os cultivos de BFV mixtos poden reducir os custos de produción de PHA e abrir novas vías biotecnolóxicas, xa que poden utilizar ácidos carboxílicos de cadea curta, derivados de procesos de fermentación, como substratos baratos e de fácil acceso e, ademais, utilizar o sol como fonte de enerxía.

Nunca antes se intentou realizar a valorización completa de FORSU utilizando cultivos mixtos BFV como tecnoloxía central para producir produtos de alto valor engadido. A maioría dos traballos realizados con cultivos mixtos de BFV céntranse no tratamento de augas residuais, e os que se ocupan da acumulación de PHA empregan case exclusivamente cultivos puros. O estudo dunha fotobiorrefinería integrada, onde se concatenan pretratamentos térmicos e procesos biotecnolóxicos para valorizar o FORSU e conseguir pechar competitivamente os ciclos do carbono e da enerxía, representa un reto aínda por explotar. Ademais, outra pregunta sen resposta son as diferentes rutas de acumulación de carbono en cultivos mixtos de BFV segundo diferentes estados redox e estrés.

Como FORSU é extremadamente heteroxéneo e pode conter compostos tóxicos, o primeiro reto desta tese é verificar que un pretratamento de hidrólise térmica pode crear un fluxo líquido co que BFV pode crecer e acumular PHA. A **Sección I** propón o estudo dunha proba de concepto de biorrefinería onde o

pretratamento por hidrólise térmica está vinculado cunha dixestión anaerobia da fracción sólida resultante e un proceso fotoheterótrofo con BFP da fracción líquida. Esta proba de concepto estudouse con FORSU real de recollida selectiva e preclasificada nunha depuradora, así como con residuos lignocelulósicos procedentes da poda do parque. Os resultados mostraron unha solubilización da materia orgánica (DQO) na hidrólise térmica de ata un 40%, con relacións DQO/nutrientes moi elevadas, sendo a temperatura máis importante que o tempo de reacción. Ademais, o pretratamento mellora a biodegradabilidade xeral do FORSU, compensando no seu potencial metanoxénico a perda da maior parte do carbono da fracción líquida. O biogás producido podería alimentar unha planta de coxeración (PCG), con resultados de xeración de enerxía térmica e eléctrica que poderían facer o proceso enerxeticamente autosustentable. En canto á asimilación da corrente líquida polo cultivo mixto de BFP, conséguese un consumo de ata o 80% do DQO con rendementos de biomasa de ata 0,5 gSSV gCOD^{-1}. Ademais, conseguíronse acumulacións de ata un 21% en materia seca de PHA, confirmando a viabilidade da proba de concepto proposta.

Para mellorar os rendementos de biomasa e a produción de PHA, sábese que as BFV asimilan máis facilmente compostos orgánicos reducidos como os ácidos carboxílicos de cadea curta (ácido acético, propiónico, butírico, etc.). Polo tanto, proponse un paso intermedio de pretratamento, a fermentación acidóxena. A **Sección II** estuda a viabilidade da co-fermentación a temperaturas termófilas dos dous compoñentes máis abundantes de FORSU: residuos de alimentos e residuos lignocelulósicos. Esta fermentación estúdase sen e con pretratamento térmico por explosión de vapor. O pretratamento por explosión de vapor mellora considerablemente a solubilización da materia orgánica e, tras ambos tratamentos, conséguense porcentaxes de solubilización de ata o 80% do DQO na corrente líquida. Ademais, a fermentación acidoxénica produce simultaneamente H_2, conseguindo nestes ensaios producións de ata 162 ± 5 mlH_2 gDQO^{-1}. Os resultados mostraron claramente un efecto sinérxico positivo sobre a eficiencia global da co-fermentación entre ambos os residuos e unha forte dependencia da distribución dos ácidos carboxílicos. Co pretratamento térmico conseguíronse valores de ata 0,58 gCOD de ácidos carboxílicos por gCOD engadido e unha acidificación do 93% (%COD referido aos ácidos carboxílicos).

Unha vez demostrado que a combinación de pretratamento térmico e fermentación acidoxénica termófila permite obter unha corrente líquida cunha

alta carga de ácidos carboxílicos de cadea curta, na **Sección III** procedeuse a estudar a fotobiorrefinería proposta (Figura R1). Un fotobiorreactor de membrana (FBRM) úsase para tratar o efluente líquido dun fermentador acidóxeno continuo alimentado con hidrolizado FORSU despois da explosión de vapor. A fracción sólida obtida despois da fermentación é recuperada por dixestión anaerobia.
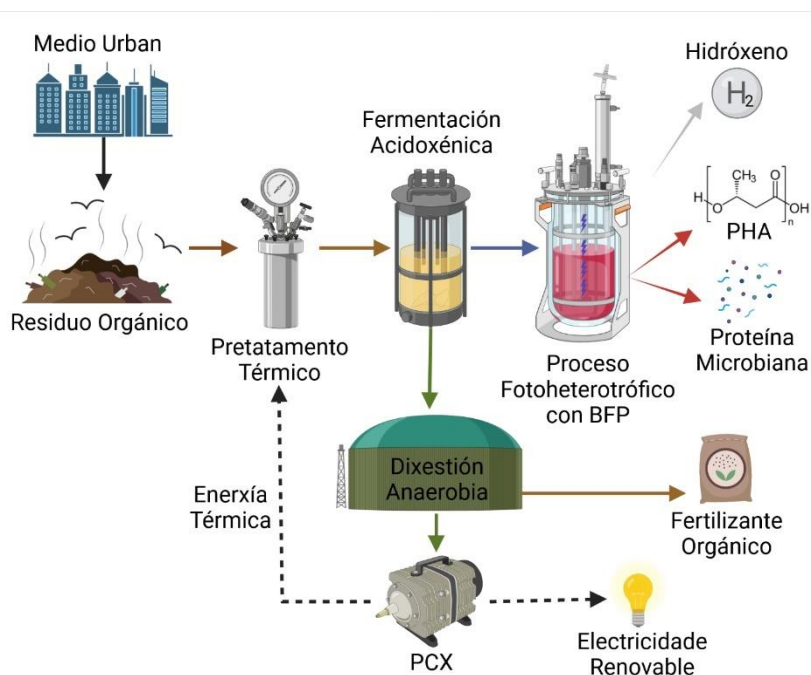
Figure R1. Representación esquemática da fotobiorrefinería de tratamento de residuos proposta.

Este estudo tenta optimizar o crecemento da biomasa e a acumulación de PHA no FBRM axustando a carga orgánica recibida polo BFV. Alcanzouse unha acumulación máxima de PHA do 42% en materia seca, o máximo obtido ata agora nun cultivo mixto de BFV alimentado con residuos. Detectáronse PHA de cadea media como o polihidroxihexanoato, con mellores propiedades físico-químicas en comparación cos PHA de cadea curta, que poderían diversificar as súas aplicacións industriais. Por outra banda, móstranse por primeira vez alternativas á acumulación de PHA: almacenamento de carbono tanto en glicóxeno como en polímeros extracelulares (PE), mentres que os electróns en exceso se derivan a H_2 mesmo en presenza de amoníaco orgánico. Por último, realizouse un estudo estatístico da evolución das comunidades bacterianas para determinar a influencia das variables ambientais na súa variabilidade,

como a variación de xéneros na FBRM tras un choque de carga orgánica ou que xéneros son os máis destacados cando PHA acumula. Finalmente, realizáronse balances de materiais e enerxía preliminares que indican que a fotobiorefinería integrada proposta pode ser enerxeticamente sostible, mentres produce 109 kg de PHA por tonelada de sólidos FORSU totais. Estes resultados son prometedores para unha implementación a maior escala dunha fotobiorrefinería baseada en BFV.

Finalmente, a **Sección IV** mostra unha revisión crítica sobre a escala do proceso máis innovador da fotobiorrefinería proposta, o proceso fotoheterótrofo con BFV. O éxito e a viabilidade económica da produción a escala industrial de biopolímeros depende principalmente de como enriquecer cultivos mixtos de BFV capaces de acumular grandes cantidades de PHA e dos principais colos de botella para a ampliación desta tecnoloxía. Coméntanse as vantaxes e inconvenientes de diferentes estratexias para a acumulación de PHA, como o festín e fame ou o festín permanente, tendo este último a clara vantaxe de poder recuperar ata o 100% de carbono (achegando o sistema á neutralidade de CO_2). unha alta conversión a PHA. En canto ao escalado, conclúese que, posiblemente, a mellor opción son os reactores ao aire libre, empregando principalmente a luz solar natural como fonte de enerxía. Non obstante, indícanse moitas estratexias que poden aumentar o rendemento do proceso, como os sistemas de captación e filtración solar e a dilución espacial da luz, así como estratexias de floculación, granulación, homoxeneización, automatización e modelaxe do sistema que poden ser útiles para o custo. redución, pero aínda deben ser investigados con maior profundidade.

No seu conxunto, este traballo propuxo e probou unha fotobiorrefinería en TRL < 3 co obxectivo de valorar FORSU en PHA e outros produtos de alto valor engadido como biogás, H_2 ou proteína microbiana. Ademais, contribúe a comprender mellor o proceso fotoheterótrofo da produción de PHA por parte de BFV para abrir o camiño para o seu desenrolo industrial.

ABSTRACT

The current Doctoral Thesis has been performed in the Department of Chemical and Environmental Technology of Rey Juan Carlos University, englobed in the research line of "Valorisation and characterization of waste materials in the framework of the circular economy", being funded in the frame of the project BIOTRES (S2018/EMT-4344 BIOTRES-CM, https://madrid.bio3project.es). Likewise, a predoctoral stay in the Research Institute for Biosciences of Universitè de Mons from May 3rd to August 3rd 2021 has been performed during the course of this Doctoral Thesis.

The transition from the current linear economy to a circular economy is one of the big challenges facing our society. One of the main drivers of this paradigmatic shift is the foreseeable depletion of material resources. Efficient resource management, therefore, becomes essential. The increase in municipal solid waste generation represents an environmental and economic cost for society, but at the same time constitutes a source of resources that cannot be squandered. More than half of this waste is a biodegradable material, known as the organic fraction of municipal solid waste (OFMSW). Indeed, this is an abundant source of low-cost carbon.

The need to secure the supply of materials and energy and minimize dependence on fossil fuels has promoted the development of biorefineries, which are analogous to oil-based refineries but use organic wastes instead of oil as feedstock. The main objective is to maximize the value derived from components and intermediates by converting them into a range of valuable bioproducts and bioenergy. Among these high value-added products, bioplastics are potential substitutes for petroleum-based plastics, ubiquitous in our lives, and cause of severe environmental and ecosystem problems. Among the possible substitutes, polyhydroxyalkanoates (PHA) are an alternative with chemical, thermal and mechanical properties similar to petroleum-based plastics but more environmentally friendly because they are biodegradable and compostable, thus a perfect product to enter the value chains of a circular economy.

Currently, the production of PHA is not economically competitive with traditional plastics. The industrial production of PHA consists of fermentative processes with pure cultures and simple feedstocks such as sugars or carboxylic acids, which makes the process more expensive due to high sterilization and raw material costs. The development of mixed culturing processes, which avoid sterilization, and waste as feedstock, has helped to reduce costs. To further decrease costs, the use of purple phototrophic bacteria (PPB) is a promising approach because of their high metabolic versatility that allows the treatment of heterogenous wastes and high biomass yields with no aeration requirements.

The most common metabolism of PPB is photoheterotrophy, through which they can use photons emitted by the sun as a source of energy and organic compounds as a source of carbon and electrons. This metabolic profile optimizes the use of organic compounds, as they do not have to derive a part for energy production, as chemotrophic cultures have to do, thus obtaining higher carbon recovery yields. In this way, the mixed PPB cultures can reduce the PHA production costs and open up new biotechnological pathways since they can use short-chain carboxylic acids derived from fermentation processes as cheap and readily available substrates and the sun as an energy source.

The complete valorization of OFMSW has not previously been pursued using mixed PPB cultures as a core technology to produce high value-added products. Most works with mixed PPB cultures involve wastewater treatment, and PHA accumulation is almost exclusively done with pure cultures. The study of complementary thermal and biotechnological pretreatments to transform the OFMSW into a suitable substrate, closing the carbon and energy cycles through anaerobic digestion processes, and optimizing PPB cultivation to achieve competitive yields of PHA and other high value-added products, represents a challenge yet to be exploited. Furthermore, the carbon accumulation pathways and the electron allocation in mixed PPB cultures according to different redox and stress states are also challenging.

As the OFMSW is highly heterogeneous and may contain toxic compounds, the first challenge of this Thesis was to ascertain that a thermal hydrolysis pretreatment could create a liquid stream upon which PPB could grow and accumulate PHA. **Section I** analyzes a biorefinery proof-of-concept where the thermal hydrolysis pretreatment links with the anaerobic digestion of the resulting solid fraction and a photoheterotrophic process of the liquid fraction

with PPB. This proof of concept was studied with a real OFMSW from selective and pre-sorted collection from a waste treatment plant and lignocellulosic waste from pruning. The results showed solubilization of organic matter (COD) in thermal hydrolysis of up to 40%, with very high COD/nutrient ratios, with the temperature being more critical than reaction time. The pretreatment also improves the biodegradability of the OFMSW, compensating the loss of the majority of the carbon present in the liquid fraction through biogas production. This biogas may feed to a cogeneration plant (CHP), resulting in thermal and electrical energy generation that might make the process energetically self-sustainable. Regarding the liquid stream assimilation by the PPB mixed cultures, up to 80% of the COD consumption were achieved with biomass yields around 0.5 gVSS gCOD^{-1}. Furthermore, accumulations of up to 21% dry mass of PHA confirmed the feasibility of the proposed proof-of-concept.

To increase biomass yields and PHA production, the PPB readily assimilate reduced organic compounds such as short-chain carboxylic acids (like acetic, propionic, butyric, and valeric). Therefore, an intermediate pretreatment step, acidogenic fermentation, is proposed in **Section II**. The feasibility of thermophilic co-fermentation of the two most abundant components of the OFMSW is studied: food waste and lignocellulosic residue. This fermentation is studied without and upon thermal pretreatment by steam explosion. The steam explosion pretreatment considerably improves the solubilization of organic matter, and after both processes, percentages of up to 80% of COD in the soluble stream are achieved. In addition, the acidogenic fermentation simultaneously produces H_2, yielding up to 162 ± 5 mlH_2 gCOD^{-1}. The results clearly showed a positive synergistic effect on the overall efficiency of the co-fermentation between the two wastes and a strong dependence on the distribution of carboxylic acids. Values of up to 0.58 gCOD carboxylic acids per gCOD added with the thermal pre-treatment and acidification extent of 93% (%COD carboxylic acids) were achieved.

Understanding that the combination of thermal pretreatment and thermophilic acidogenic fermentation provides a liquid stream with a high loading of short-chain carboxylic acids, the modified photobiorefinery is proposed in **Section III** (Figure R1). A membrane photobioreactor (MPBR) was used to treat the liquid effluent coming from a continuous acidogenic fermenter fed with the OFMSW hydrolysate obtained after the steam explosion. The solid fraction of the fermentate is recovered through anaerobic digestion.
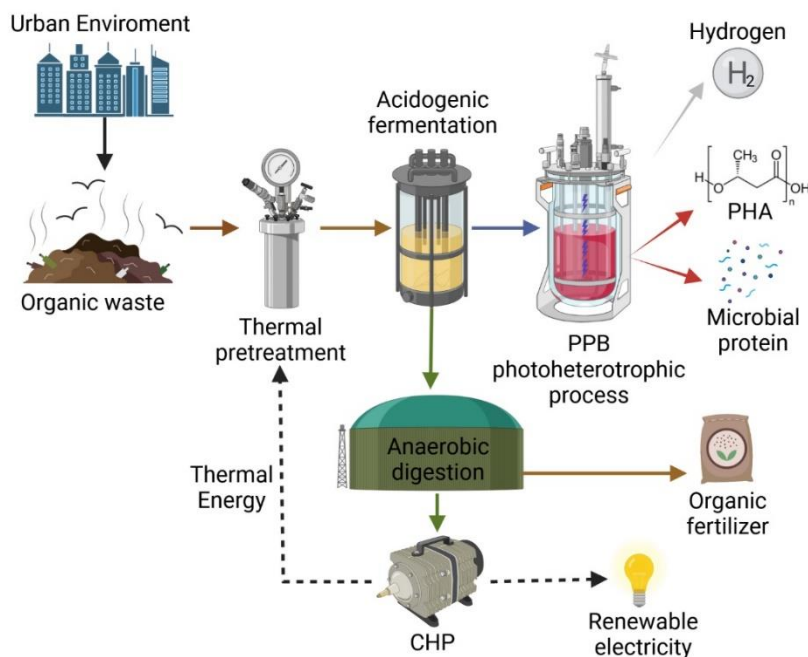
Figure R1. Graphical abstract of the proposed waste valorization photobiorefinery.

In this work, we attempted to optimize biomass growth and PHA accumulation in the MPBR by adjusting the organic load of the PPB. A maximum PHA accumulation of 42% (gPHA gVSS^{-1}) was achieved, which is the highest obtained so far in a mixed culture of waste-fed PPB. Medium-chain PHA such as polyhydroxyhexanoate have been detected, which can theoretically improve their physicochemical properties compared to short-chain PHA and could diversify their industrial applications. Furhtermore, the section shows, for the first time, alternatives to PHA accumulation: carbon storage in both glycogen and extracellular polymers (EPS) while deriving excess electrons in H$_2$ even in the presence of ammonium. Finally, a statistical study about bacterial population dynamics has been carried out to establish the environmental variables with the most significant influence on the variability of these bacterial communities. Finally, preliminary material and energy balances were carried out indicating that the proposed integrated photobiorefinery can be energetically sustainable. This work demonstrates the importance of acquiring a thorough understanding of the carbon accumulation and electron allocation strategies of PPB under stressful environmental conditions and shows promising results for a larger scale implementation of a PPB-based photobiorefinery.

Finally, **Section IV** critically reviews the scale-up of the most innovative process of the proposed photobiorefinery, the phoheterotrophic process with PPB. The success and economic viability of an industrial-scale production of biopolymers depend mainly on enriching mixed PPB cultures capable of accumulating large amounts of PHA and the main bottlenecks for the scale-up of this technology. The advantages and disadvantages of feast-and-famine vs. permanent feast strategies are discussed, with the latter having advantages such as the possibility of recovering up to 100% carbon (bringing the system close to CO_2 neutrality) with high conversion to PHA. It is concluded that outdoor reactors, with mainly natural solar illumination, are probably the best option for the scaling-up of this technology. However, many strategies can increase the performance of the process, such as solar collection and filtration systems and spatial light dilution, flocculation, granulation, homogenization, automation, and system modeling strategies that can be useful for cost reduction, but still, need to be further investigated.

Overall, this work has proposed and tested at a TRL < 3 photobiorefinery with the aim of valorizing FORSU into PHA and other high-value-added products such us biogas, H_2 and microbial protein. Furthermore, it helps to better understand the photoheterotrophic process of PHA production using PPB to pave the way for its industrial deployment.

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LIST OF ABREVIATIONS

AD = Anaerobic Digestion

ATP = Adenosine Triphosphate

BMP = Biochemical Methane Potential

B_o = Biodegradable Extent

CA = Carboxylic Acids

CHP = Combined Heat and Power

COD = Chemical Oxigen Demand

CrI = Cristaline Index

CSTR = Continuosly Stirred Tank Reactor

DRX = X-Ray Diffraction

ED = Entner-Doudoroff

EMP = Embden-Meyerhof-Parnas

EPS = Extracellular Polymers

FTIR = Fourier Transform Infrared Spectroscopy

FW = Food Waste

GC = Gas Cromatography

GHG = Greenhouse Gases

H_E = Hidrolisis Extent

HRT = Hydraulic Retention Time

HTC = Hydrothermal Carbonization

HTL = Hydrothermal Liquefaction

K_h = Hydrolysis Constant

K_M = Specific Phototrophic Activity

LW = Lignocellulosic Waste

MMC = Mixed Microbial Cultures

MPBR =Membrane Photobioreactor

MSW = Municipal Solid Waste

NADH = Nicotinamide Adenine Dinucleotide

OFMSW = Organic Fraction of Municipal Solid Waste

OLR = Organic Loading Rate

ORP = Oxidation and Reduction Potential

PAR = Photosynthetically Active Radiation

PPS = Pentose-Phosphate Shunt

PCA = Principal Component Analysis

PHA = Polyhydroxyalkanoate

PHB = Polyhydroxybutirate

PHH = Polyhydroxyheaxanoate

PHV = Polyhydroxyvalerate

PPB = Purple Phototrophic Bacteria

q_{EPS} = EPS Production Rate

q_{GLY} = Glycogen Production Rate

q_{PHA} = PHA Production Rate

q_s = Specific Substrate Uptake Rate

RDA = Redundant Analysis

SCCA = Short-Chain Carboxilic acids

SCOD = Soluble Chemical Oxigen Demand

SRT = Sludge Retention Time

TCOD = Total Chemical Oxigen Demand

TKN = Total Kjeldahl Nitrogen

TP = Total Phosphorus

TS = Total Solids

TSS = Total Suspended Solids

UASB = Upflow Anaerobic Sludge Blanket

VS = Volatile Solids

VSS = Volatile Suspended Solids

Y_{EPS} = Extracelular Polymer Yield

Y_{GLY} = Glycogen Yield

Y_{PHA} = PHA Yield

$Y_{X/S}$ = Biomass Yield

PUBLICATIONS, ORAL AND POSTER COMUNICATIONS

Publications

Allegue, L. D., Puyol, D., and Melero, J. A. (2020). Food waste valorization by purple phototrophic bacteria and anaerobic digestion after thermal hydrolysis. Biomass and Bioenergy, 142, 105803. https://doi.org/10.1016/j.biombioe.2020.105803
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Gamboa, M., **Allegue, L. D.**, Puyol, D., Dugour, J., and Melero, J. A. (2022). Environmental life cycle assessment of polyhydroxyalkanoates production by purple phototrophic bacteria. *In preparation.*

Other publications

Allegue, L. D., Ventura, M., Melero, J. A., Baptiste, L. and Puyol, D (2022). Release of crotonic acid in anaerobic photoheterotrophs: a new metabolic mechanism for attaining redox homeostasis in high organic conditions. *In preparation.*

Allegue, L. D., Molina, R., Ventura, M., Melero, J. A., and Puyol, D. (2022). Electron and carbon allocation as the key for modelling polyhydroxyalkanoates production by purple phototrophic bacteria mixed cultures. *In preparation*.

Oral Comunications

Allegue, L. D., Puyol, D., and Melero, J. A. (2019). Integrated photo-biorefinery for OFMSW valorisation to high value-added products and energy in *3rd ANQUE-ICCE International Congress of Chemical Engineering*, Santander, Spain.

Allegue, L. D., Puyol, D., and Melero, J. A. (2020). Nonlinear parameter and uncertainty estimation of anaerobic digestion kinetics on hydrothermally pretreated lignocellulosic waste in *Conference on Biogas Production and Digestate Valorisation* (online), Madrid, Spain.

Allegue, L. D., Puyol, D., and Melero, J. A. (2020).Valorización de biorresiduos urbanos mediante el acoplamiento de procesos de hidrólisis térmica, digestión anaerobia y fotosíntesis anaerobia in XIV *Conference on Biomethanisation of solid urban waste* (online), Barcelona, Spain.

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biorefinery in *8th International Conference on Sustainable Solid Waste Management* (online)*,* Thessaloniki, Greece.

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Allegue, L. D., Ventura, M., Melero, J. A., and Puyol, D. (2022). Organic load affects carbon and electron allocation in OFMSW photobiorefinery of purple phototrophic bacteria for PHA accumulation in 9*th International Conference on Sustainable Solid Waste Management,* Corfu, Greece.

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Poster Presentation

Allegue, L. D., Puyol, D., and Melero, J. A. (2019) Foto-biorefinería para la conversión de residuos orgánicos urbanos en productos de alto valor añadido in *IWA Young Water Professionals Spain Conference 2019*, Madrid, Spain.

Zamora, P., Marín, E., **Allegue, L.D.,** Villamil, J., Martínez, F., Melero, J.A., Puyol, D., Rogalla, F., Monsalvo, V. (2020). DEEP PURPLE: Purple Phototrophic Bacteria for the development of low-cost treatment of mixed urban bio-wastes **in** *IWA World Water Congress and Exhibition*, Copenhagen, Denmark.

Projects

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CHAPTER 1: STATE OF THE ART

1.1. OVERVIEW: CIRCULAR ECONOMY AND CLIMATE CHANGE

Climate change is the greatest challenge facing our society in this century. Anthropogenic emissions of Greenhouse Gases (GHG) have led to an increase in atmospheric CO_2 by 47% above pre-industrial levels, which is higher than the natural increase over the past 20,000 years (NASA, 2021a). The increase of the CO_2 concentration in the atmosphere contributes to the greenhouse effect, altering the planet's climate and ecosystems and increasing the temperature during the last decades by more than 1°C (NASA, 2021b). Therefore, the international environmental policies are seeking a universal agreement to keep global warming below a critical threshold, that is, to limit temperature rise in the next decade to 1.5 °C (IPCC, 2018), thus avoiding a biosphere breakdown. This objective entails a radical reduction of emissions through the decarbonization of the production model, migrating to a zero-emission economy. Emission reduction policies generally focus on decreasing GHG generation from energy production, but they should also limit the number of raw materials used in manufacturing since they account for 45% of total GHG emissions (Ellen MacArthur Foundation, 2019; IRP, 2019). In a context of a growing population, increasing demand for scarce resources, and climate burden, changing our current "make - use - discard" production model to one based on the precepts of the circular economy is imperative.

A transition to a circular economy requires a drastic shift in our approach to the production and consumption of products. The circular economy concept is based on optimizing manufacturing through product reuse, life extension, and recycling loops of its components (Kalmykova *et al.*, 2018). Figure 1.1 shows a diagram of the resource flows in a circular economy, illustrating the potential for extending the life of both renewable flows and finite resources while minimizing losses or negative externalities to a minimum. Improving resource efficiency and material reduction should be an essential element of climate policy (Rizos *et al.*, 2019). According to a Club of Rome's report, estimating the effects of a shift to a circular economy in 5 EU countries (Finland, France, the Netherlands, Spain, and Sweden) could lead to a reduction of CO_2 emissions of up to 75% (Wijkman and Skånberg, 2016). For example, applying this production model to the food industry could reduce emissions by 49% (Ellen MacArthur Foundation, 2019). Another main driver of the circular economy is reducing and recycling waste, whereby the European Commission's Waste Package estimated that it could reduce 443 million tons of GHG between

2014 and 2030 (European Commission, 2014). Conservation and closed loops in resource value chains are implicit in the circular economy. Resources from one sector are converted into raw materials in another sector, attempting to maintain the quality and status of the resources or even upgrading them (upcycling) (Korley *et al.*, 2021). The increasing urban expansion of industrialized countries has led to a steady increase in Municipal Solid Waste (MSW) generation, which is a complex problem for our society but can be turned into valuable resources due to the scarcity and high prices of certain raw materials.
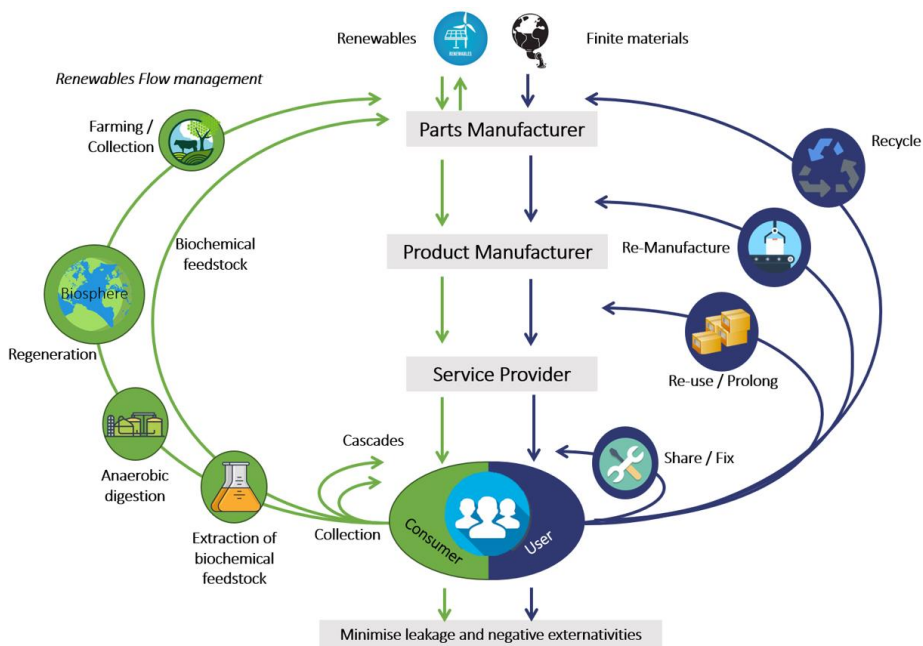
Figure 1.1 Resource flows through a value chain in a circular economy Adapted from Ellen MacArthur Foundation, (2019).

1.2. MUNICIPAL SOLID WASTE IN A CIRCULAR ECONOMY

The management of MSW challenges the EU as a critical element to realize a circular economy. The EU 27 has produced 505 kg of municipal waste per capita in 2020 (Eurostat, 2020). Therefore, it is essential to reduce the percentage of MSW deposited in landfills and increase waste recycling. However, the reduction of landfilling was only 58% in 2020 compared to 1995 but this value must be lower than 10% (Figure 1.2). On the other hand, 27% of MSW is managed by incineration (no distinction is made between incineration

and incineration with energy recovery) and 18% by composting. Only 30% of MSW is being recycled, far from the EU target of 55% by weight settled by 2025. In the Waste Framework Directive and the Landfill Directive, high emphasis is placed on the **organic fraction of municipal solid waste (OFMSW)**, which is a key element in the planning of the sustainable MSW management system (Council Directive 1999/31/EC on the Landfill of Waste, 1999). OFMSW must be collected separately from 2023 onwards, a major step in the establishment of a circular bioeconomy in the EU (Sherwood, 2020).

Year	Target
2009	Less than 50% of MSW to landfill
2016	Less than 35% of MSW to landfill
2023	Biowaste must be collected separately and not mixed with other waste
2025	Phasing out landfilling by 2025 for recyclable (including bio) waste Reclycling of 55% by weight of MSW Reducing food waste generation by 30%
2030	Reclycling of 60% by weight of MSW Reducing food waste generation by 50% 65% of the packaging waste has to be recycled
2035	Less than 10% of MSW to landfill Reclycling of 65% by weight of MSW

Figure 1.2 EU legislation targets MSW management. Source: (Council Directive 1999/31/EC on the Landfill of Waste, 1999; EC. Com. 397, 2014; European Commission, 2020; Eurostat, 2020)

The OFMSW includes food and kitchen waste from households, restaurants, supermarkets, food processing plants, and garden and park waste. Depending on their origin, they may contain different proportions of readily biodegradable simple organic matter like sugars, starch, proteins, and lipids, more recalcitrant compounds like lignocellulosic matter, or unwanted fractions like plastic packaging or inorganic materials (Moretti *et al.*, 2020). Biowaste

percentage in the overall MSW varies significantly among European countries (Figure 1.3), averaging 37% (EEA, 2020). These variations rely on the urbanization level and the country-specific data collection and reporting system. For example, in Spain the percentage is higher, being in the range of 40 to 60% of the total MSW (MAGRAMA, 2015). In a circular economy, organic waste is directed to treatment options that use the waste as a source of valuable resources such as nutrients, organic substances, and energy.
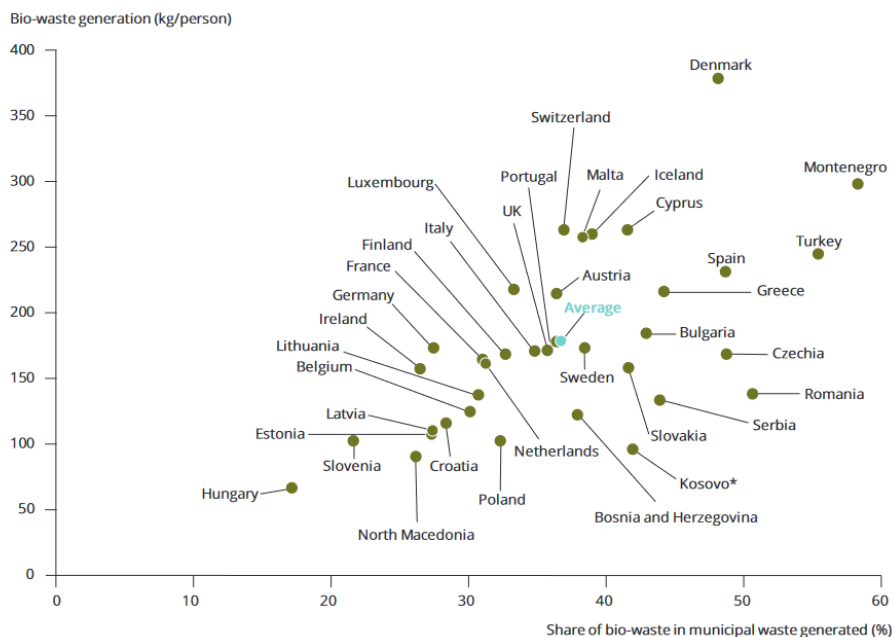
Figure 1.3 OFMSW generation per person and share of bio-waste in MSW generated by country, 2017. Source: (EEA, 2020).

The separated collection of the OFMSW is beneficial when valorizing the organic matter adequately. The two most common treatments for OFMSW are composting and anaerobic digestion (AD). Composting generates a humic product through aerobic oxidation that can be used as a fertilizer or soil improver, while in AD anaerobic consortia conduct a series of syntrophic chemical reactions that produce a digestate and biogas that can be used to generate electricity or heat. In any case, both alternatives are considered low value and do not take advantage of the high potential of this resource. However, the EU has defined a treatment hierarchy that prioritizes the valorization of organic wastes into biomaterials rather than compost or energy (Figure 1.4), thus defining the scope of the biorefineries for OFMSW (European Commission, 2018). Indeed, the fundamental contribution of biorefineries to

the concept of circular economy is the 'pyramidal approach,' where the extraction and production of an extensive portfolio of high added-value products have greater priority than bioenergy production.
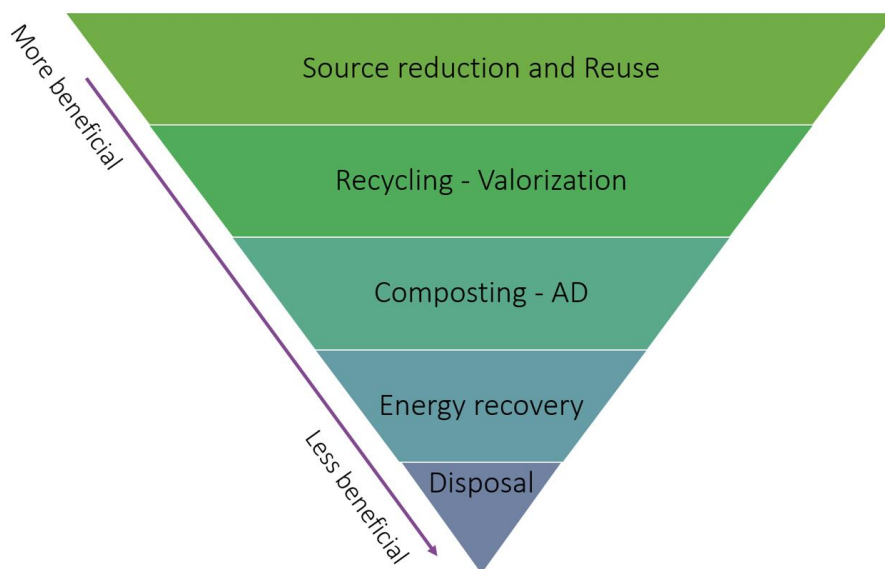
Figure 1.4 Waste hierarchy adapted from European Commission, (2018, 2020).

1.3.   BIOREFINERIES: THE WORKHORSE OF THE BIOECONOMY

In the transition to a circular economy, we must reassess our approach to environmental management and the exploitation of the planet's resources, embracing waste as a valuable resource and closing the loops around industrial ecosystems. Biorefineries combine innovative technologies to produce biofuels, high value-added products, and bioenergy to achieve this goal from different organic wastes. This feedstock is of significant interest since it does not compete with the food industry and the need for arable land is not required. The most challenging aspect of this kind of feedstock is its seasonal variability, heterogeneity, moisture content, and the recalcitrance of the organic material compared to the fossil-based feedstock that has traditionally been used to manufacture fuels and chemicals in conventional refineries.

The most widely used platforms for the transformation of organic waste into value-added products are (Holtzapple *et al.*, 2022):

1. Thermochemical platform: It consists of thermal degradation of recalcitrant biomass such as: pyrolysis, a thermal process in absence of oxygen that generates mainly bio-oil but also biochar; gasification, a thermal process in presence of low levels of oxygen that yields syngas (CO_2 + H_2) as main product; hydrothermal carbonization (HTC) where wet biomass is converted into a rich-carbon solid product called hydrochar; and hydrothermal liquefaction (HTL) to obtain as primary product a biocrude. These thermochemical processes have as main drawback the requirement of high energy input (even higher for pyrolysis and gasification where the biomass needs to be previously dried).

2. Sugar platform: In this biological approach exogenous enzymes hydrolyze polysaccharides (cellulose and hemicellulose) to simple sugars that are subsequently fermented to the corresponding alcohols. Nowadays, this technology has low yields which makes it not competitive against the fermentation process using starch and edible sugars (Tonini and Astrup, 2012).

3. Carboxylate platform: A set of processes where a mixed culture of microorganisms uses endogenic enzymes in a fermentation process to obtain carboxylic acids with a carbon range from C1 (formate) to C8 (octanoate) or even higher. These compounds can later be transformed into fuels, heat, electricity and high value-added products such as bioplastics or biochemicals (Agler *et al.*, 2011).

The carboxylate platform has the advantages of being resilient and environmentally sound, having higher product yields (Holtzapple *et al.*, 2022), and being industrially applicable and scalable (Jones *et al.*, 2021).

1.3.1. THE CARBOXYLATE PLATFORM

AD is still the core process technology of modern biorefineries, with the potential of waste stabilization, biogas production, recovery of chemical building blocks for the carboxylate platform and nutrients. The methane produced in the biogas can be used directly to produce heat and electricity in a combined heat and power (CHP) plant or upgraded to natural gas quality by removing water vapor and CO_2 and fed into the natural gas grid (Kumar and Samadder, 2020). In recent years, the upcycling of biogas obtained through the valorization of CO_2 by using microalgae or the cultivation of methanotrophs for their use as feed ingredients has also been gaining ground (Tsapekos *et al.*, 2021). However, separating the processes occurring within the AD to maximize

the production of carboxylates represents an advantageous configuration, as it does not require the dissolution of feedstock and maximizes the concentration of products and nutrients for recovery in downstream processes, as well as avoids leakage and the potential emission of methane into the atmosphere if biogas is not handled adequately (Kleerebezem *et al.*, 2015). Therefore, a thorough understanding of the different phases within the anaerobic degradation process of organic waste is key for developing the carboxylate platform.

The carboxylate platform describes an anaerobic fermentation process (either named as dark fermentation or acidogenic fermentation) that produces carboxylates as building blocks to generate higher-value products (Figure 1.5). Carboxylates are organic acids having at least one carboxyl group and up to 20 carbon atoms. Depending on the pH, they can be in the form of carboxylate salts or carboxylic acids. Short-chain carboxylic acids (SCCA) have one to eight carbon atoms, commonly referred to as volatile fatty acids (VFA), when reduced to 6 carbon atoms.
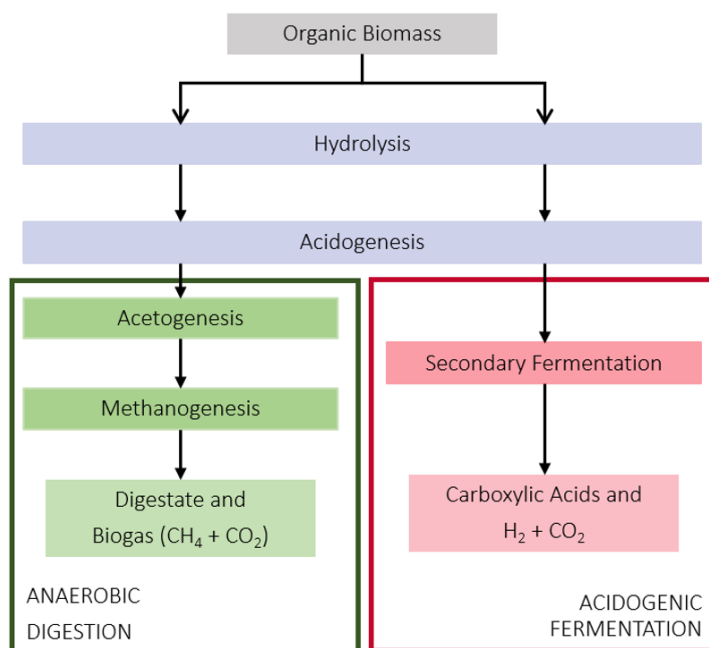
Figure 1.5 Schematic representation of the main differences between AD and acidogenic fermentation biochemical pathways.

Figure 1.5 shows the main different biochemical pathways between AD and acidogenic fermentation for converting complex organic matter into metabolic

compounds. The anaerobic fermentation used on a carboxylate platform has three steps: hydrolysis, primary fermentation or acidogenesis, and secondary fermentation; however, in normal anaerobic digestion, it consists of four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Kumar and Samadder, 2020). Therefore, the main difference between the carboxylate platform and the AD is that the disposal of electrons by methanogenesis is partially or entirely arrested.
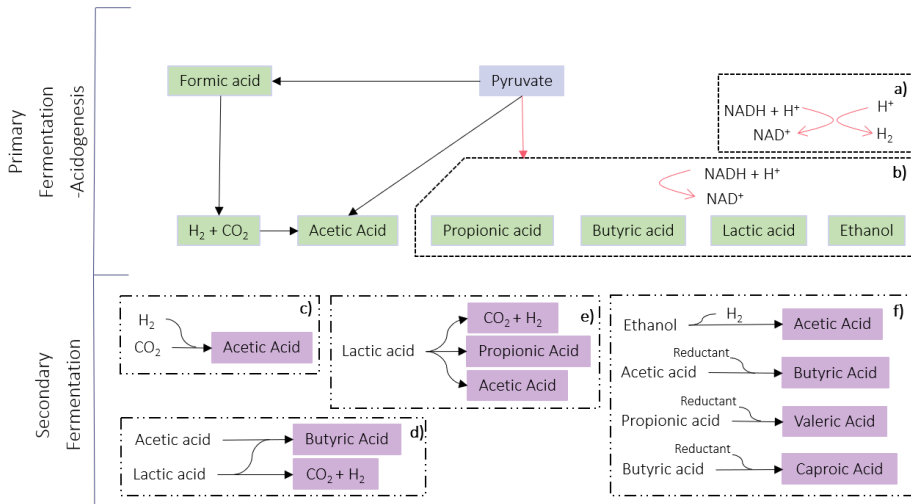
Figure 1.6. Biological pathways in the carboxylate platform. Subsequent conversions by primary and secondary fermentation reactions with undefined mixed cultures. Adapted from Agler *et al.*, (2011).

The first two phases are the same for AD and acidogenic fermentation; firstly, in the hydrolysis phase, complex organic substances that bacteria cannot directly utilize are broken down into soluble compounds by the action of acidic hydrolytic enzymes. For example, proteins are transformed into amino acids, lipids into fatty acids, and polysaccharides into monomers and oligomers. Figure 1.6 shows the biochemical pathways linked to acidogenic fermentation. During the primary fermentation of sugars, substrates are converted to pyruvate, which results in the production of NADH and H^+. All equivalents must be re-oxidized via H^+ reduction by: (a) NADH oxidation; or (b) NADH oxidation via reduction of pyruvate or its oxidized organic derivatives, depending upon the hydrogen partial pressure. At increasing hydrogen partial pressures, the flow of electrons from NADH shifts from H_2, acetic acid, and CO_2 production towards the formation of increasingly reduced fermentation products (McInerney and Bryant, 1981). CO_2 and H_2 are produced in pyruvate oxidation

or via dehydrogenation of formic acid. Figure 1.6 shows that the products of primary fermentation can react further through several secondary fermentation reactions: (c) autotrophic homoacetogenesis; (d) lactic acid oxidation to butyric acid (acetic acid and H^+ as an electron acceptor); (e) lactic acid reduction to propionic acid (oxidation to acetic acid for energy conservation); (f) ethanol oxidation and chain elongation of carboxylates with a reductant (ethanol, lactic acid, hydrogen, etc).

The population of methanogenic archaea must be inhibited to promote the production of SCCA. The most widespread strategies to accomplish this are targeted inoculation, thermal pre-treatments, pH shocks, or inhibitor supplementation (Dahiya *et al.*, 2015; Xie *et al.*, 2014; Yu *et al.*, 2014). However, the most suitable strategy to reduce operating costs and obtain an effective start-up is to inhibit methanogenesis by overloading the methanogenic population. High organic loading rates (OLR) or shortened hydraulic retention time (HRT) cause imbalances between the methanogenic and acidogenic populations, accumulating SCCA in the reactor, consequently causing a decrease in pH and buffer capacity, which inhibits methanogenesis (Rajagopal *et al.*, 2013). Upon methanogenesis inhibition, the excess of reductants are released through SCCA production, lowering the energy state compared to other pathways (e.g., ethanol). In most anaerobic habitats, acetic, propionic, and butyric acid are the most commonly produced SCCA, but many other secondary routes are possible.

Different groups of anaerobic bacteria produce SCCA, and a thorough understanding of anaerobic metabolism is essential to maximize their potential and comprehend the diversity of SCCA obtained in the process. Most of these bacteria are chemotrophic and obtain energy through the oxidation of high-energy compounds (e.g., sugars) to lower energy compounds (e.g., acetic acid). To maintain the redox balance, electrons released in these oxidations are transferred enzymatically by electron carriers (e.g., NADH, NADPH or FADH), which donate electrons to electron acceptors (oxidants). The primary process is anaerobic catabolism, whose fundamental challenge is to exploit electron acceptors to capture energy while maintaining redox balance during the oxidation of substrates. Two processes are used for this challenge: fermentation, which uses organic electron acceptors, or anaerobic respiration, which uses inorganic electron acceptors (e.g., CO_2, NO_3, SO_4, Fe_3^+). Electron acceptors have a lower reduction potential than O_2, thus anaerobic bacteria have a lower energy yield for growth and higher retention of chemical energy

in catabolic products than aerobic bacteria (which release CO_2 as the primary end product of metabolism). Predicting the catabolic products generated in a mixed anaerobic culture with heterogeneous substrates is highly challenging. It depends primarily on the structure of the microbial community and the identity and concentration of the donors and acceptors, which define the thermodynamics and kinetics of the metabolic reactions. These variables are the main drivers in determining which biochemical pathways are the most advantageous in individual anaerobic habitats.

1.3.2. PRETREATMENT

Pretreatment is a fundamental step in the acidogenic fermentation of solid organic wastes. The hydrolysis of complex organic matter is a rate-limiting step, especially in recalcitrant substrates such as lignocellulosic residues (Romero-Cedillo *et al.*, 2017). Hence, these pretreatments are necessary to speed up the initial hydrolysis step. Although studies on OFMSW pretreatment specifically focused on SCCA production are scarce some insights can be inferred from studies carried out in AD, even if they are not optimized for SCCA production. The different types of pretreatments can be classified into chemical, physical, and biochemical (or enzymatic). These pretreatments result in the acceleration of the hydrolysis stage and the yield of SCCA and hydrogen production. Overall, the drivers to select the appropriate pretreatments are operation costs, enhancement efficiencies, and desired final products.
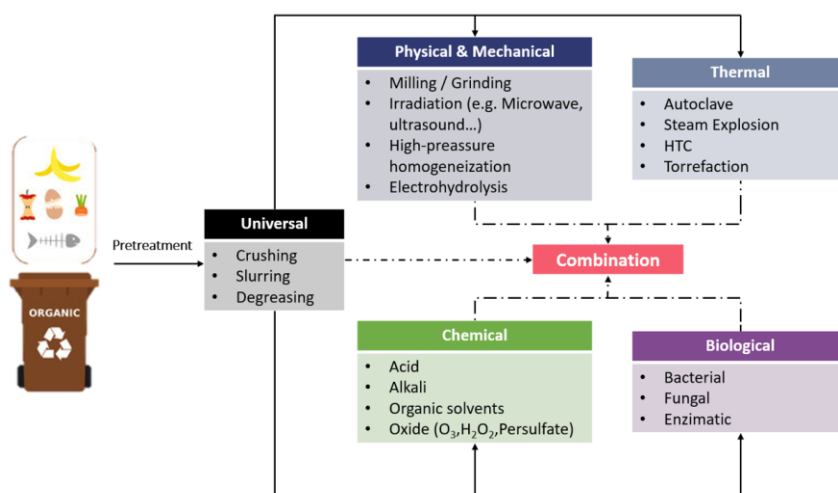
Figure 1.7. Ilustration of the various pretreatment methods for organic solid waste. Adapted from Zhang *et al.* (2021).

As shown in Figure 1.7, after sorted OFMSW is crushed to reduce volume, and conventionally mechanically pretreated, other pretreatment methods are frequently used to improve the biological treatment effect. As an expensive but indispensable step in OFMSW resource utilization, appropriate pretreatment or even combined pretreatment methods should be selected from multiple perspectives such as biomass substrate composition, subsequent biological treatment methods, and economic feasibility. For example, chemical pretreatments can be effective, but have several disadvantages such as toxic compound generation. Moreover, they are not environmentally sound pretreatments and require recovery or neutralization treatments (Ramos-Suarez *et al.*, 2021). On the other hand, biological pretreatments are environmentally friendly, but their use on substrates of variable composition such as OFMSW is often discouraged (Plácido and Zhang, 2018). Hence, chemical and biological pretreatments are not very spread at the industrial level.

Among the physical pretreatments, both mechanical and thermal ones are the most studied and most technologically mature, but other emerging technologies are being implemented at both research and industrial levels, such as high-pressuring machines, microwaves, and ultrasound devices.

Mechanical pretreatment such as screening, meshing, or grinding improves the surface area, enhancing biodegradability and solubility, and they are typically used as a first step before thermal pretreatments. In AD, particle reduction accelerates the accumulation of SCCA (Romero-Cedillo *et al.*, 2017). In particular, it can increase the amount of acetic acid produced from food waste (FW) (Izumi *et al.*, 2010). This type of pretreatment allows particle reduction for better handling of waste in subsequent processes and has the advantage of requiring low energy and being simple to implement. The disadvantages are that it does not degrade lignin, present in lignocellulosic waste (LW), it does not remove pathogens or significantly increase the soluble chemical oxygen demand (SCOD) dissolution, and it has high maintenance requirements (Cesaro and Belgiorno, 2014).

Conventional hydrothermal pretreatment relies on temperature and reaction pressure control, although the latter is usually the endogenous pressure that increases with increasing temperature in a closed vessel like an autoclave. Hydrothermal pretreatment at a wide range of temperatures (55-200 °C) and times (5-60 min) has been considered, depending on the optimal

pretreatment temperature on the type of substrate, use, etc. Earlier thermal pretreatment was used to improve the dewaterability and digestibility of sludge in the AD field. According to EU regulation EC1774/2002 organic, solid waste should be sterilized or pasteurized at least for 1 h at 70 °C before or after AD (Ariunbaatar *et al.*, 2015). Thermal pretreatment improves the AD process by solubilizing refractory particles (Ariunbaatar *et al.*, 2015), deflocculating macromolecules, improving dewaterability (Jin *et al.*, 2016), disinfecting by sterilization (Li and Jin, 2015), and reducing exogenous pollution. However, thermal pretreatment may also inactivate methanogenic archaea present in the feedstock. Generally, the effect of thermal pretreatment is measured in terms of soluble chemical oxygen demand (SCOD), SCCA, and biogas production. SCOD increases significantly due to the degradation and dissolution of insoluble organic compounds such as carbohydrates, lipids, and proteins (Ahmed *et al.*, 2021).

The effects of hydrothermal pretreatment have been studied on three main components of OFMSW such as kitchen waste, fruit and vegetable waste, and waste activated sludge, resulting in a considerable decrease of viscosity and an increase of SCOD, mainly sugars and soluble proteins (Liu *et al.*, 2012) at 175 °C and 60 min. A recent study on hydrothermal pretreatment of dewatered sewage sludge validated that a pretreatment at 170 °C achieved an efficiency of 0.59 gCOD$_{SCCA}$ gCOD^{-1} (Chen *et al.*, 2021). However, temperatures higher than 150°C promote the formation of refractory compounds via Maillard reactions, i.e., interactions between sugars and amino acids at high temperatures that can inhibit anaerobic processes (Tyagi *et al.*, 2018). In fact, temperatures as low as 100 °C are sufficient to improve the hydrogen production yield due to the effective suppression of methanogenic communities (Dong *et al.*, 2010). Therefore, evaluating physical pretreatment conditions is as vital as the waste composition analysis concerning the possible formation of not degradable by-products requiring proper monitoring systems.

Hydrothermal pretreatment is an environmentally friendly process because it does not use chemicals and has zero emissions. The integration of thermal pretreatment with acidogenic fermentation of OFMSW could have several potentially positive outcomes for sustainable production: increased process stability, increased specific SCCA and hydrogen yields, maximized substrate availability for the microbial community, reduced energy requirements during the fermentation process; reduced HRT, downsizing reactor volume and reduced remaining biosolids landfill use (Habarakada and Babel, 2020). One of

the main drawbacks of this technology is the high pressure and high temperature, leading to energy-demanding and costly treatment. One strategy to overcome this problem is to use steam explosion, which is usual for lignocellulosic biomass and is becoming common for OFMSW, mainly in the presence of a nearby AD plant.

Steam explosion

Steam explosion is one of the few pretreatment technologies that have evolved to the point that pilot-scale and process equipment are commercially available. The most established commercial technology is the CambiTHP © process, and the schematic representation of the process is presented in Figure 1.8. Briefly, it consists of heating of the organic waste with saturated steam, followed by a sudden decompression of the pressurized system in a flash steam-heat recovery system and a standard size multi-reactor. This process requires a relatively low steam amount as it is recycled from the flash tank to the pulper where the feed is stored (Abu-Orf and Goss, 2021). It usually operates at 160 °C and 6 bar for 30 min and achieves high cell disintegration, where organic solids are dissolved in the water, such as proteins and carbohydrates, which are disintegrated into oligo- and monosaccharides and amino acids. In addition, this feedstock is well sterilized, eliminating the risk of contamination by pathogens. Steam explosion is the most widely used chemical-physical pretreatment method for lignocellulosic biomass, but it has already been installed in food and sludge co-digestion plants in China, Norway, South Korea, and Sweden (Ahmed *et al.*, 2021). It has also been applied for source-separated OFMSW in Lillehammer, Oslo where 14,000 tonnes/y are treated and a 70% reduction of volatile solids (VS) is achieved (Barber, 2016).
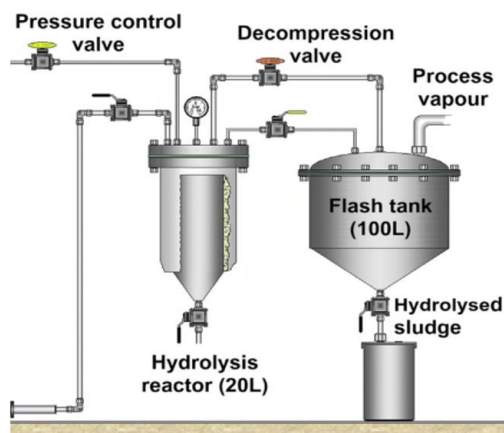
Figure 1.8 Schematic representation of a typical steam explosion reactor.

The economic feasibility analysis of thermal pretreatment methods for OFMSW processing at the pilot-scale or full scales are limited in the literature. One of the keys to achieving a sustainable operation is the energy self-sufficiency of the process (Clauser *et al.*, 2021). Several pilot scale studies have already demonstrated a positive energy balance of steam explosion pretreatment with a CambiTHP © system combined with AD on several different organic wastes (Cano *et al.*, 2014; Díaz *et al.*, 2021). In any case, the energetic, economic and environmental feasibility of a hydrothermal pretreatment process can be improved by incorporating the use of renewable energies (e.g., solar), segregation of waste at source, co-digestion approach, and avoidance of high-temperature thermal pretreatment of carbohydrate and protein-rich substrates (Fan *et al.*, 2018). **An important task in this doctoral Thesis has been to study more in depth how hydrothermal pretreatment influences in the production of SCCA coupled with acidogenic fermentation, as well as a better understanding of how it affects each main element of the OFMSW to optimize time and temperature parameters.**

1.3.3. OPTIMIZING SCCA PRODUCTION

The most important parameters to be optimized during acidogenic fermentation are the methanogenic inhibition, the waste characteristics, inoculum and microbial population, temperature, OLR and HRT. These factors affect both the yield and the type of SCCA produced. Figure 1.9 summarizes the strategies for their optimization, and briefly explained below:

Methanogenesis inhibition	Waste characteristics	Inoculum	Temperature	OLR and HRT
• pH control: Below 6 or above 7.5 • Short HRT • Methane inhibitors: MES, BES or lumazine	• Particle size or lignocellulosic residue: • Pre-treatments • C/N ratio: Co-fermentation	• Inoculum acclimatisation • Optimisation of S/I ratio • Heat treatment to inhibit methanogens	• Thermophilic process (45-65ºC) favours high OLR and solid state fermentations, as it increases the hydrolysis step	• High OLR and low HRT • HRT: 4-12 days • Progressively increase OLR

Figure 1.9. Workflow for optimization of operating parameters during acidogenic fermentation.

a) Methanogenesis inhibition. The optimum pH for methanogens is 7.0 while acidogenic bacteria can handle wider pH ranges (5-11), which leaves room for acidic or alkaline fermentations. Slightly acidic fermentations improve hydrolysis due to increased activity of the hydrolytic bacteria, although alkaline fermentation can also be beneficial as they improve digestibility by dissolving

lignin and can offer buffering capacity. However, alkaline addition can add high operational costs, whereas an acidic environment can be maintained by accumulating SCCA and providing a substrate close to pH neutrality. The growth of methanogens is slower than acidogens. Hence, a shorter HRT could also achieve the wash-out of methanogens, avoid consumption of SCCA and improve the treatment capacity (Yin, *et al.*, 2016).

b) Waste characteristics. Although the literature on acidogenic fermentation mainly focuses on optimizing hydrogen production, we can draw key conclusions for this process. For example, waste composition affects the overall yield. High-complex protein waste, such as FW, has the potential to produce higher concentrations of SCCA (Ramos-Suarez *et al.*, 2021). However, LW (between 50%-90% cellulose, hemicellulose, and lignin content) require pre-treatment to be digested properly (Jin *et al.*, 2016). The composition of the feedstock can be modified by mixing different substrates, in which case the process is known as co-fermentation. Co-fed substrates are known to improve performance due to synergistic effects such as helping to dilute toxic compounds present in the feedstocks and improving the nutrient balance, particularly the C/N ratio (Farmanbordar *et al.*, 2020; Soomro *et al.*, 2020). The presence of minerals in the fermentation medium is also very important to enhance microbial growth, although their effect on SCCA production is poorly studied (Zhang and Shen, 2006).

c) Inoculum. SCCA yield and distribution for a given substrate, are in essence a result of the microbial community and its activity. Microbial community studies can help understand the acidogenic fermentation process and the effect of fermentation conditions. Acidogenic species can be introduced through inoculation/seeding, sometimes from eutrophic waters, but most studies use AD digestate/sludge as the inoculum (Ramos-Suarez *et al.*, 2021). Nevertheless, AD sludge contains methanogens that convert the acetic acid into methane, therefore acclimatization of the inoculum for extended periods (Plácido and Zhang, 2018) or thermal pretreatment of the inoculum to inhibit methanogens (Blasco *et al.*, 2020) have been studied as possible strategies to improve the process. Regarding the process stability, sporulation must be controlled or avoided. During the process, sporulation decreases the substrate consumption rates and productivity and loses the microbes' autocatalytic capacity (Hawkes *et al.* 2002), increasing the dominance of bacteria populations that do not have sporulated (Hawkes *et al.*, 2002).

d)   Temperature. This parameter has a significant effect on the yield and the SCCA produced. Typically, fermentation is classified according to the operating temperature as psychrophilic (<25 °C), mesophilic (25-45 °C), or thermophilic (>45 °C). In general, thermophilic conditions have a significantly higher accumulation of SCCA compared to mesophilic conditions (He *et al.*, 2012; Yin, *et al.*, 2016). In addition, thermophilic temperatures can enhance the hydrolysis of solid residues improving overall digestibility, but pH has a more significant influence on this than temperature (Garcia-Aguirre *et al.*, 2017). Temperature can also potentially influence the nature of SCCA produced. However, the findings are inconsistent at present (Zhang *et al.*, 2009), probably due to limited knowledge on variables interactions.

e)   OLR and HRT. Low HRT (4 to 9 d) favors the production of acetic and butyric acids, promoting the dominance of acidogenic bacteria and prevents the growth of methanogenic microorganisms (Renaudie *et al.*, 2021). In contrast to HRT, high OLR values stop methane production and promote acidogenesis. Increasing OLR above the AD threshold (7 gVS L^{-1} d^{-1}) results in higher SCCA concentrations but lower yields. Therefore, a compromise between yield and concentration must be found. In addition, the optimal OLR will vary depending on the substrate, temperature, etc. In summary, the optimal process conditions must be studied in situ, depending on the substrate used, the cost analysis and the SCCA ratio to be obtained.

Bioreactor Configuration for acidogenic fermentation

The bioreactor configuration influences the hydrodynamics and impacts the liquid-gas mass transfer phenomena, thus producing changes in the population of dominant microorganisms and enzymatic/metabolic changes. Figure 1.10 shows the most commonly used bioreactors for acidogenic fermentation. The two common technologies used in the anaerobic production of SCCA from waste are attached growth and suspended growth.

The two most used attached growth reactors are the packed and fluidized bed reactors. In packed bed reactors the biomass grows and adheres to porous packing material, such as alumina-based ceramic cubes and granular activated carbon (Beccari *et al.*, 2009), inserted into the reactor. This retains the biomass in the reactor, thus alleviating biomass washing. However, the packed bed reactor will become clogged with waste containing high concentrations of suspended solids. To avoid clogging, a fluidized bed reactor has been developed since in this type of reactor, the biomass grows attached to a small solid

medium, such as sand, which remains in suspension by the upward movement of the fluid (Lee *et al.*, 2014). However, these types of reactors do not support high organic loadings (OLR) and have low volumetric activities, so suspended biomass reactors are more common.
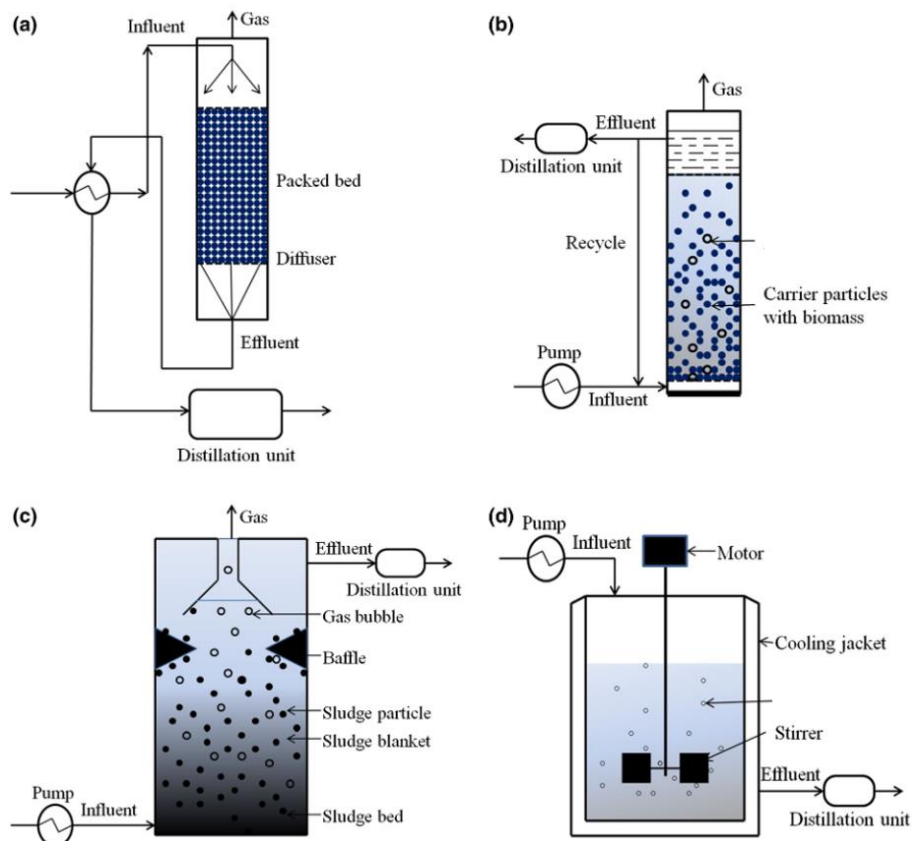
Figure 1.10. Various types of reactors used in SCCA fermentation (Bhatia and Yang, 2017). a) Packed bed reactor, b) fluidized bed reactor, c) upflow anaerobic sludge blanket reactor (UASB), and d) continuous stirred tank reactor (CSTR). Workflow for optimization of operating parameters during acidogenic fermentation.

Suspended growth technology allows the biomass to grow freely in suspension. Examples of reactors operated based on suspended growth technology are the upflow anaerobic sludge blanket (UASB) reactor and the continuous stirred-tank reactor (CSTR). The most common industrially used reactor is the UASB reactor, which consists of an upstream gas-liquid-solid separator and relies on the formation of dense and readily settleable biomass called granules (Wang *et al.*, 2007). These granules are retained in the reactor by sedimentation, forming a sludge blanket at the bottom of the reactor reducing the required reactor volume and increasing the efficiency of the

process. The biggest drawback of the UASB reactor is a longer start-up period if the inoculum is not yet granulated (Jung *et al.*, 2011).

On the other hand, the operation of CSTR is the most straightforward, achieving complete mixing of waste and biomass, improving mass transfer (Show *et al.*, 2011). This can be approximately achieved by well-designed impellers, baffles and reactor shape. This mixture contributes to attaining saturation in the liquid phase, and the CO_2 and H_2 produced are transferred to the gas phase. Therefore, headspace clarification is also decisive for the chosen bioreactor since liquid saturation and the partial pressure of H_2 gas negatively affect the conversion reactions of NADH to H_2 by hydrogenases (Hallenbeck, 2005). A major problem with CSRT is the wash-out of biomass, but this problem can be fixed easily by a gravity settling clarifier used to separate and to recycle the biomass from the effluent. **When designed and operated properly, a CSTR is ideal to mix waste and microbes thoroughly in the presence of suspended solids in the waste**.

Parameter homogenization

In order to standardize and improve communication channels between the academy and industry, the units of the key variables must be well defined, as the disparity in literature prevents proper conclusions from being drawn. In AD processes it is common to use VS percentages for the characterization of substrate consumption, however in fermentation processes significantly less VS will be converted to CO_2 (and negligible amounts to CH_4) and instead, most are converted to SCCA. COD units are a better indicator to define SCCA yields and concentrations to compare these values with other soluble components present in the fermentation broth. SCCA composition is highly dependent on substrate and operational parameters, so standardization of product concentrations in terms of COD allows comparison between a range of variables. The proportions and yields of each SCCA present should also be provided, as this will allow a better understanding of the conversion and distribution of the product.

A common measure of fermentation performance is the **acidification extent**, which measures the equivalent COD of the SCCA over the SCOD in the broth. It is a good indicator of fermentation performance as it measures the purity within the aqueous solution obtained, which is extremely important in the downstream process. Whether this aqueous solution is used for further biological steps or recovery and purification of the SCCA obtained, the maximum possible acidification should be sought. However, it is not a complete overview of the fermentation yield; for example, the hydrolysis extent is

another indicator that considers the ratio of SCOD increased in the reaction as well as the equivalent COD of the H_2 produced. It is also common to indicate the obtained SCCA concentrations which, although an interesting parameter, depends on the OLR or substrate concentration and therefore makes it difficult to compare between experiments.

1.3.4. RECOVERY AND APPLICATIONS

The shortage of industrial SCCA-based processes is partly attributed to the difficulty in recovering the SCCA from the fermentate as well as low product yields. Individual SCCA are fundamental platform chemicals to produce esters, ketones, aldehydes, alcohols, and alkanes, therefore, the recovery process must selectively target the SCCA over other fermentation broth components including water, and increase their concentration in the product stream. Membrane extraction, electrodialysis and filtration have been the most researched recovery techniques (Ramos-Suarez *et al.*, 2021), but are still far from reaching industrial scale. The energy demand, and the number of unit operations required to achieve the desired product stream, and the fermentation broth conditions should be considered when designing the recovery process, which at the moment is not cost-effective (Greses *et al.*, 2020).

To bypass this bottleneck, direct use of the fermentation products in other bioprocesses is a promising alternative, although some physical processes like centrifugation to separate solid and liquid streams are necessary. The bioconversion of mixed organic acids entails hydrogen production, high added-value products like PHA, microbial protein, chain elongation products, or can allow nitrogen and phosphorus removal from wastewater (Ramos-Suarez *et al.*, 2021). **The implications of this platform and the different options available will be discussed throughout this research.** The production of bioplastics from PHA is one of the most attractive sectors in recent years, with considerable research into its production from waste (Rodriguez-Perez *et al.*, 2018). Nevertheless, this technology is still looking to advance to become economically viable, as PHA is not yet competitive with equivalent petrochemical plastics such as polyethylene (Ramos-Suarez *et al.*, 2021). Nevertheless, purple phototrophic bacteria (PPB) are attractive candidates for PHA production because, among other features, they can obtain energy from infrared light instead of oxygen, reducing operating costs.

1.4.   NEXT STEP: THE PHOTO-BIOREFINERY

Photosynthesis is the main supplier of carbon and energy required for the synthesis of the organic compounds that drive plant growth and development (Hussain *et al.*, 2021). Phototrophic microorganisms can be a powerful tool for the efficient conversion of the virtually unlimited supply of solar energy into bioenergy and renewable resources (Goh *et al.*, 2019; Tanvir *et al.*, 2021). The photosynthetic efficiency of these microorganisms (~10%) is much higher than that of terrestrial biomass (1.8-2.2%) (Ooms *et al.*, 2016). Among these microorganisms, two of the most studied are microalgae and cyanobacteria, which have 10 times the lipid content and require a quarter of the cultivation time of terrestrial plants (Ghosh *et al.*, 2016). In addition, they are highly adaptable to dynamic environmental conditions and do not compete for arable land (Zhu *et al.*, 2014). These microorganisms have the ability to feed on heterogeneous feedstocks such as some waste streams and accumulate high value-added bioproducts and bioenergy, which makes them excellent candidates to be the central element of a biorefinery using light as an energy source: a Photobiorefinery.

Phototrophic microorganisms capable of producing oxygen during the process of photosynthesis are known as oxygenic phototrophs (e.g., cyanobacteria, green algae), otherwise they are known as anoxygenic phototrophs (e.g., green and purple bacteria). Most oxygenic phototrophs absorb solar energy from the visible light region of the solar spectrum (400-700 nm), while anoxygenic phototrophs can photosynthesize with light from the visible and infrared regions, as shown in Figure 1.11. Oxygenic phototrophs require more input energy for photosynthesis (shorter wavelength light) compared to anoxygenic phototrophs (longer wavelength light) due to the inverse relationship between photon energy and wavelength (Chen and Blankenship, 2011). Anoxygenic phototrophs contain bacteriochlorophyll(s) instead of chlorophyll-like oxygenic phototrophs and require only a single type of photochemical reaction center (RCI or RCII) to carry out photosynthesis (Zeng *et al.*, 2015). Most species of the phyla *Chlorobi*, *Chloroflexi,* and *Acidobacteria* contain a unique light-harvesting pigment known as a chlorosome to absorb sunlight at 740-750 nm, while **purple phototrophic bacteria (PPB)** harvest sunlight from the infrared region (800-1020 nm) of the light spectrum (Hanada, 2016). Furthermore, they are the only species capable of absorbing light beyond 1000 nm wavelength (Chen and Blankenship, 2011), which would give access to 19% additional photon flux compared with standard photosynthetically active radiation (PAR). Moreover, it allows them to grow without competing for the same light source, making them microorganisms

with unique features. Therefore, **PPB has been the microorganism object of study in this research.**
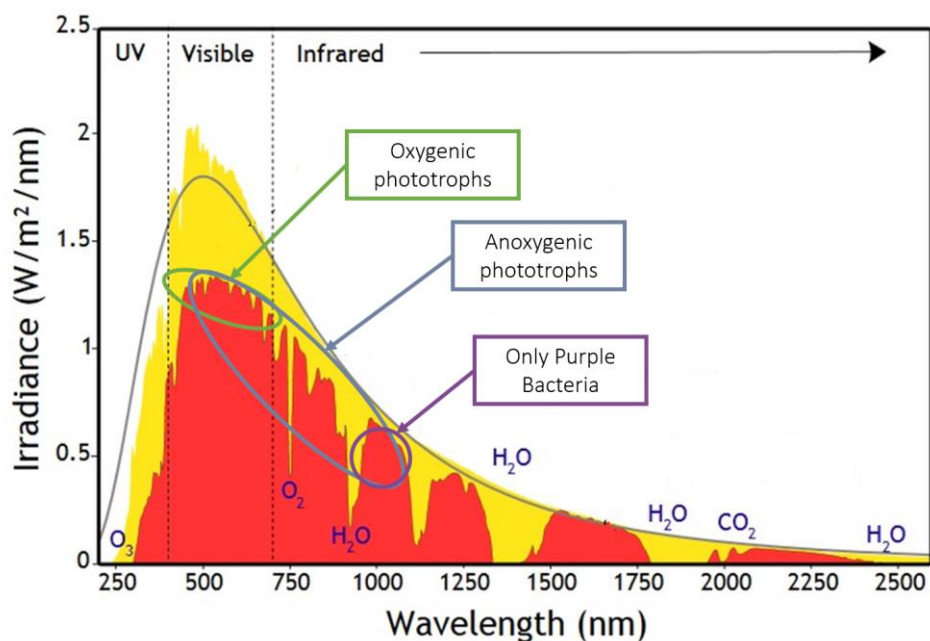
Figure 1.11. Standard solar radiation spectrum. In yellow, the spectrum is high in the atmosphere. In red, is the spectrum at sea level.

1.5. PURPLE PHOTOTROPHIC BACTERIA

PPB are a diverse group of anoxygenic, phototrophic, facultative anaerobes that inhabit aquatic and terrestrial environments. PPB have typically been classified according to their sulfur tolerance and utilization into purple sulfur and non-sulfur bacteria. Purple sulfur bacteria can oxidize sulfur to sulfur globules, which are stored intracellularly, while purple non-sulfur bacteria deposit them outside the cell (Hunter *et al.*, 2006). Overall, PPB are one of the most metabolically diverse types of microorganisms known on earth. Table 1.1 shows a summary of all possible metabolisms of PPB. In presence of light and an anaerobic environment, the two most common metabolisms are photoautotrophy and photoheterotrophy. Both use light as an energy source, while the former uses CO_2 as a carbon source, and the latter uses organic carbons (Capson-tojo *et al.*, 2020). Under these conditions, most catabolic energy comes from light absorbed by bacteriochlorophylls (BChl) and carotenoids (pigments required for light-harvesting and photosynthetic growth). In the presence of oxygen at high concentrations, suppression of BChl

and carotenoids occurs (Yue *et al.*, 2015), which causes the loss of the usual color of PPB and shifts the metabolism towards chemotrophy using O_2 as an electron acceptor (Dubbs *et al.*, 2000). However, it is also possible that a mixed metabolism occurs where PPB obtain part of the energy from chemical catabolism and part from light in a mixed process of photo-fermentation. **This great metabolic flexibility allows PPB to be a perfect tool for treating a wide variety of heterogeneous wastes.**

In the last decade, there have been many studies on the use of PPB for wastewater treatment (Cao *et al.*, 2020). The abundant carbon (C), nitrogen (N), and phosphorus (P) in wastewater are highly dissipated resources. However, the current trend follows the circular economy in wastewater management, away from conventional treatments and focuses on resource recovery, where PPB is a perfect tool (Puyol *et al.*, 2017). PPB can tolerate wastewater with ample organic strength from different sources such as domestic wastewater (Hülsen *et al.*, 2016), poultry (Hülsen *et al.*, 2018), food processing (Chitapornpan *et al.*, 2012), brewery (Peng *et al.*, 2018) or slaughterhouse (De la Vega *et al.*, 2022) among others. PPB have also been used for soil remediation, polyphosphate accumulation, or monitoring environmental stress (Capson-tojo *et al.*, 2020). Nevertheless, the processes have not yet reached an industrial scale.

Table 1.1. Main metabolic modes of PPB. Adapted from Puyol et al. (2019).

Metabolism	Process	e⁻ donor	e⁻ acceptor	Energy source	Carbon source	Redox conditions
Photo-organo-heterotrophy	Heterotrophic photosynthesis	Organic	CO_2 (fixation), H^+ (H_2 production)	Infrared light	Volatile fatty acids, alcohols, sugars	Anaerobic
Photo-litho-heterotrophy		H_2	CO_2 (fixation)			
Photo-litho-autotrophy	Autotrophic photosynthesis	$S^x/S_2O_3^{2-}$	CO_2 (fixation)		CO_2	
	Photoanaerobic Fe^{2+} oxidation	Fe^{2+}				
	Photoanaerobic H_2 oxidation	H_2				
Chemo-organo-heterotrophy	Aerobic oxidation			Chemical	Organic	Anoxic
	Fermentation	Organic	Organic			
	Denitrification	Organic	NO_3^-			
Chemo-litho-autotrophy	Nitrification	NH_4^+	O_2		Volatile fatty acids, alcohols, sugars, proteins	Aerobic
	Halophilic S^{2-} oxidation	S^{2-}	O_2		CO_2	

Compared to the usual chemoheterotroph technologies, the most significant advantage of PPB's technology is their ability to generate energy via photophosphorylation growing through anaerobic phototrophy. This results in:

1. <u>Increased biomass yields</u>. Since there is no need for ATP generation from chemicals, PPB biomass yields on simple substrates can reach values up to 1.0 gCOD gCOD$_{removed}^{-1}$ (Puyol *et al.*, 2017), which is better compared to the yields commonly achieved in activated sludge systems (0.5 gCOD gCOD$_{removed}^{-1}$, Henze *et al.*, 1997). Moreover, when the substrate is more reduced than the biomass, yields higher than 1 gC gC^{-1} are possible by simultaneous CO_2 fixation.

2. <u>No aeration is needed</u>. In this case, the energy comes from light, which can be supplied cost-free by solar illumination. The economic efficiency of artificial lighting is still a matter of debate. According to estimates in a current review article from Capson-tojo *et al.* (2020), even using NIR LED lamps (800-1000 nm), the energy cost of biomass production would be pretty high (1.7 € kg$_{biomass}^{-1}$). This cost could be reduced by increasing the biomass concentration in the bioreactors through biomass recirculation techniques and optimizing the volumetric irradiance.

3. <u>Effective selection of mixed PPB cultures in non-sterile environments for heterogeneous waste treatment</u>. As discussed in previous sections, PPB absorbs at wavelengths between 805 and 1035 nm, with no competition in that spectrum from other microorganisms. These features provide this technology with clear advantages for its application, but the optimal high value-added products must fit the economic balances when scaling up. **The key bottlenecks of the scale-up of the photoheterotrophic process will be discussed in this Doctoral Thesis.**

1.5.1. HIGH ADDED-VALUE PRODUCTS

In addition to the main growth metabolic pathways, PPB can undertake accumulation or side processes that allow them to better survive in stressful environments, such as the discontinuous presence of carbon or nutrients. Figure 1.12 summarises the products that can be extracted from PPB and some strategies for increasing their production. In excess of organic matter and absence of essential nutrients, PPB can accumulate carbon mainly as polyhydroxyalkanoates (PHA) (Fradinho *et al.*, 2016). However, they can also accumulate glycogen (Fülöp *et al.*, 2012) or even produce extracellular polymers (EPS) in aggregated growing mode (Stegman *et al.*, 2021). PPB can also accumulate the excess of critical nutrients like P and S as polyphosphates form (Liang *et al.*, 2010) or as intracellular globules after sulfide oxidation

(Weissgerber *et al.*, 2014), respectively. PHA, polyphosphate, and S globules all have in common that they serve as sinks for reducing redox potential and PHA and S can be used also as alternative electron donors. Another key mechanism of PPB is the release of excess electrons as H_2 to maintain redox homeostasis via nitrogenase in a low ammonium environment (Hädicke *et al.*, 2011). H_2 production by PPB using waste as substrate in photo fermentation processes has already been extensively studied (Ghosh, *et al.*, 2017), even by coupling acidogenic fermentation with photofermentation to maximize H_2 production (Rai and Singh, 2016). Although it is a promising technology as a stand-alone process, there is not yet a widespread scale-up of the technology in outdoor reactors (Sagir and Alipour, 2021). Bottlenecks such as slow productivity and low light conversion efficiency in the cells remain critical problems for the scaling-up of this technology (Tiang *et al.*, 2020). What is certain is that the scale-up of these products will have to consider all these mechanisms and possible production synergies in a PPB biorefinery.
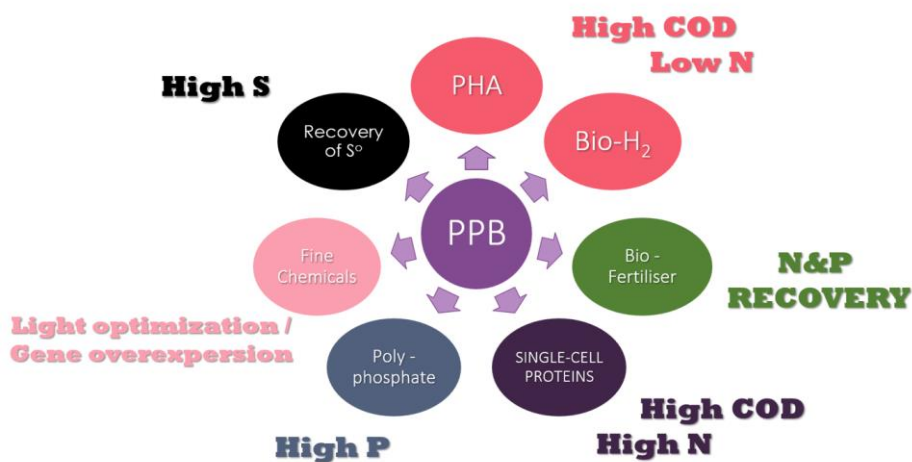
Figure 1.12. Possible strategies for producing different high value-added products using the metabolic versatility of PPB. Adapted from Puyol *et al.* (2019).

Other high-value products can be obtained from PPB according to different cultivation strategies. If the substrate contains high levels of nutrients (especially N, P, and K), PPB biomass can serve as an organic fertilizer or biofertilizer exhibiting a high capability to recover and recycle C and N (Zarezadeh *et al.*, 2019). With high C and N substrates, PPB cultivation can be oriented to protein production. PPB can accumulate high amounts of protein (>70%) with high-quality amino/fatty acid profiles (Alloul *et al.*, 2021). Finally, PPB can be used to extract fine chemicals such as carotenoids (Kuo *et al.*, 2012), 5-aminolevulinic acid (5-ALA) (Kars and Alparslan, 2013), or coenzyme Q10 (He

et al., 2021). Carotenoids are pigments used in cosmetics and as food coloring agent formulation. 5-ALA is a non-protein amino acid that can be used as an herbicide or an active component for the medical industry. The production of both products can be optimized by increasing the light intensity in PPB cultivation (Yu *et al.*, 2021). Coenzyme Q10 is a type of ubiquinone mainly used in the medical and cosmetic fields. Several attempts have been made to optimize its production via gene editing in pure PPB cultures (Lu *et al.*, 2013). The major problem for the commercialization of these products is the high cost and complexity of their extraction (Bogacz-Radomska and Harasym, 2018), which usually involves using environmentally toxic solvents.

1.6. POLYHYDROXYALKANOATES (PHA): A BIODEGRADABLE PLASTIC

In recent years the research on PPB is shifting from water treatment to resource recovery, mainly PHA. The main reason is that plastics are nowadays widely used as synthetic polymers, mainly due to their resistance to chemical and physical degradation. However, at the end of their life cycle, the synthetic plastics' resistance to degradation has caused one of the world's major environmental problems: plastic pollution (Heidbreder *et al.*, 2019). Replacing petroleum-based polymers with bio-based polymers is a potential solution that produces significantly lower carbon emissions and energy production requirements (Gironi and Piemonte, 2011). Bio-based polymers can be subdivided into three types, plant-based (TPS), polymerized bio-monomers (i.e., PLA, polyimides, polyurethanes, poly(butylene succinate) (PBS), bio-PE, among others), and extracted bio-polymers (PHA) (Meereboer *et al.*, 2020). PLA and PHA are biodegradable (de Castro *et al.*, 2021), specifically, PLA is compostable but not marine biodegradable like PHA (Meereboer *et al.*, 2020). Biodegradability is essential if we want to move towards circular economy practices. Moving towards bio-based biodegradable polymers allows for a more sustainable option by implementing a cradle-to-cradle approach (Braungart *et al.*, 2007), where the output of biodegradation becomes the production input for the same polymer in a reasonable time frame within the biological cycle.

PHA are becoming important due to their many advantages such as their biodegradability, biocompatibility (Chen and Wu, 2005), controllable thermal and mechanical properties (Laycock *et al.*, 2014), as well as PHA molecular weight diversity ranging from several tens of thousands to several millions Daltons (Rodriguez-Perez *et al.*, 2018). PHA are accumulated intracellularly and assembled into hydrophobic spherical inclusions and have the biological

function of carbon and energy storage (Reddy *et al.*, 2003). Many types of PHA are known, being the most well-known poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV). Both are short-chain PHA and represent the most basic forms commercially available (Leong *et al.*, 2014). However, many other types of PHA can be classified by their chain length:(i) a short chain length of 3-5 carbons; (ii) a medium chain length of 6-14 carbons; and (iii) a long chain length of 15+ carbons. Examples of the most common medium-chain PHA are polyhydroxyhexanoate (PHH) or polyhydroxyoctanoate (PHO) (Meereboer *et al.*, 2020). The properties of PHA depend on the chain length and combination of monomers present. In general, copolymers decrease the degree of crystallinity and melting temperature and increase the extension at break. However, in particular, the combination of short-chain PHA (PHB and PHV) with medium-chain PHA, such as polyhydroxyhexanoate (PHH), lead to polymers with elastic properties that increase their value in the industry, as they can be used as additives for medical and pharmaceutical applications (Pereira *et al.*, 2019).
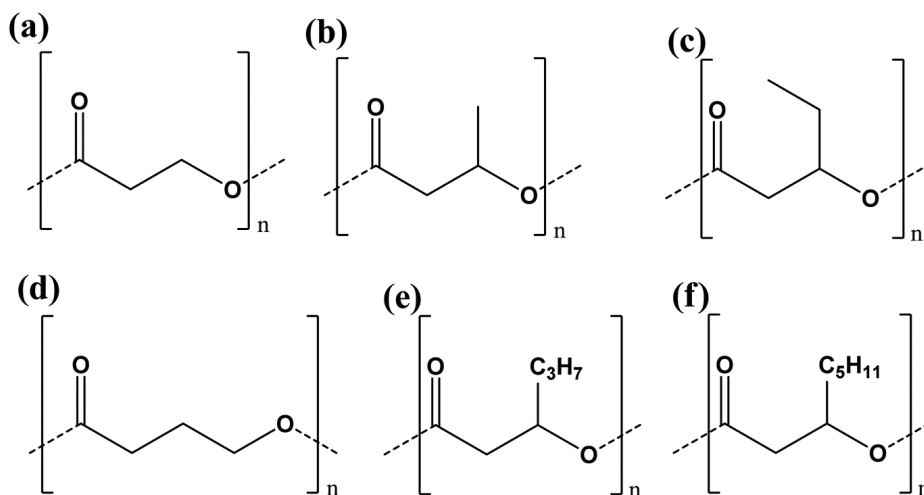
Figure 1.12. Different PHA functional components. (a) 3-Hydroxypropionate (HP), (b) 3-hydroxybutyrate (HB), (c) 3-hydroxyvalerate (HV), (d) 4-hydroxybutyrate (4HB), (e) 3-hydroxyhexanoate (Hx), and (f) 3-hydroxyoctonaote (HO) chemical structures.

1.6.1. CURRENT INDUSTRIAL PHA PRODUCTION

While it is challenging to estimate the current volume of PHA produced industrially, according to the Global Bioplastics Market Report 2020, annual PHA production exceeded 10,000 t annually, with a 20–30% yearly growth rate (Bhola *et al.*, 2021). Industrially, PHA is synthesized through fermentation, using pure and engineered aerobic heterotrophic microorganisms fed with

relatively pure plant-derived substrates like sugars and starch, among others, in batch reactors. The typical strategy is dynamic feeding in two steps: a selection reactor (feast phase) and a storage reactor (famine phase). Industrial-scale PHA are manufactured by various companies worldwide, for example, BioMatera (Toronto, Canada), Metaboli (Woburn, USA), Procter and Gamble Co., Ltd. (Cincinnati, USA), Tianjin Green Bioscience Co., Ltd. (Tianjin, China) Bio-on (Italy), Biocycle PHB Industrial SA (Serrano, SP, Brazil) and Goodfellow Cambridge, Ltd. (UK) (Palmeiro-Sánchez *et al.*, 2022). However, the price of PHA remains relatively high (1.7 - 3.5 € kg PHA^{-1}) and is 2 to 4 times more expensive than petroleum-based plastics (Tan *et al.*, 2021). In any case, the EU under the umbrella of the Green Deal and more specifically in the Directive on single-use plastics (Directive (Eu) 2019/904 of the European Parliament and of the Council of 5 June 2019 on the Reduction of the Impact of Certain Plastic Products on the Environment, 2019) and the Packaging Waste Directive (DIRECTIVE (EU) 2018/852 on Packaging and Packaging Waste, 2018) considers bioplastics indispensable for the achievement of climate targets, as they can be collected along with the organic waste and treated in a circular and environmentally sustainable way.

The main bottlenecks in industrial PHA production must be firstly identified to reduce production costs. The main one is the substrate cost and the sterilization required in the use of pure cultures, which can account for more than 50% of the production cost (Kourmentza *et al.*, 2017). The other is the cost of the downstream process: both the cost of extracting and purifying the PHA, which can account for up to 30% of the total cost (Fernández-Dacosta *et al.*, 2015). The most commonly used strategies to reduce these costs are using mixed microbial communities (MMC) as biocatalysts for PHA generation under non-sterile conditions and applying a variety of low-value substrates, such as industrial and municipal waste and by-products (Novelli *et al.*, 2021). These processes, mainly with aerobic microorganisms, achieve high PHA dry mass contents (0.5 - 0.9 g_{PHA} $g_{DryBiomass}^{-1}$) and substrate conversion yields up to 32% (Koller, 2018). **Nevertheless, the production of PHA with mixed PPB cultures is gaining great attention in the last years** (Monroy and Buitrón, 2020) **and shows several features that make this strategy very exciting and promising.**

PHA accumulation routes via PPB are complex and vary depending on various parameters, including bacteria strain, carbon substrate, and metabolic pathway. Photoheterotrophic production of PHA is the predominant route from which attractive advantages over aerobic microorganisms are foremost:

1.   PPB can deliver up to 90% PHA yields on substrates, almost three times higher than aerobic yields (Fradinho *et al.*, 2019).

2.   PPB does not require aeration, as they obtain their energy through IR illumination.

3.   PPB can accumulate PHA while growing, and nutrient availability drives the accumulation process (Fradinho *et al.*, 2016). Thereby, PPB can accumulate PHA in a permanent carbon feast regime, eliminating standby production times and simplifying the operation to a single reactor, as opposed to the commonly used feast/famine regime that involves two stages: In the first stage, an abundant carbon source is provided to favor the production of biomass, and in the second stage, the carbon source is limited to stimulate PHA accumulation (Fradinho *et al.*, 2019).

However, the outstanding performance of PPB have not yet been fully exploited, and further optimization of PHA accumulation is part of the research of this Doctoral Thesis with the purpose of helping scaling up and commercializing this technology.

1.6.2. PHA APPLICATIONS

As indicated, depending on the monomeric composition, different properties of the designed PHA can be obtained. The polymer can be hard and crystalline or elastic and rubbery. These properties will designate the future application of the polymer. As an example, pure PHB is highly crystalline with a melting temperature of 180°C making it brittle and stiff (Koller, 2018). In contrast, the different copolymers that can be formed and medium-chain PHA are materials with more elastic properties, high elongation at break, low crystallinity and glass transition temperatures, less mechanical resistance, and low melting points; hence, they are more versatile materials and more desirable for industrial applications (Shahid *et al.*, 2021). PHA has unique properties that give it an edge over other biopolymers in this field. It has improved barrier properties (sufficient oxygen and water transmission rates) and higher mechanical strength than PLA (Mathuriya and Yakhmi, 2017). It also has lower acidity and reduced bioactivity, as well as greater proliferation, stronger calcium deposition, and fibrillar collagen synthesis than PLA or polyglycolic acid (PGA) (Ali and Jamil, 2016). PHA have applications in the following sectors:

-   <u>Medical and farmaceutical sector</u>. PHA can be used as artificial heart valves, blood vessels, cartilage or tendons, nerve conduits, esophagus

replacements, bone replacement, surgical sutures, porous microspherical implant-scaffolds for microsurgery, and many other applications (El-malek *et al.*, 2020; Moradali and Rehm, 2020; Muneer *et al.*, 2020).

-   Packaging industry. It can be used for the production of bottles, cosmetic containers, bags, and utensils, for example (Mozejko-Ciesielska *et al.*, 2019; Muneer *et al.*, 2020). It can also be converted into biofilms for the food industry, as it has the necessary properties (Topuz and Uyar, 2020).

-   Agricultural sector. PPB with PHA are proposed as bacterial inoculants to improve nitrogen fixation in plants and also can be used to make soil friendly compostable greenhouse films, grow bags, and protection nets (Muneer *et al.*, 2020).

-   Other sectors. PHBV copolymer is also used to synthesize a polymer gel electrolyte for use in high-density lithium batteries (Dall'Asta *et al.*, 2017). It also can be used as a biofuel precursor (Montiel-Corona and Buitrón, 2021).

1.6.3. Mechanisms of PHA synthesis by PPB

The PPB preference for acetic acid as a substrate for PHA production has been repeatedly reported due to its easy assimilation into the metabolic pathway for Acetyl-CoA synthesis, a precursor to PHA (Fradinho *et al.*, 2014). PPB uses different metabolic pathways to assimilate acetic acid, such as glyoxylate, citramalate, and ethyl malonyl-CoA pathways. Figure 1.13 depicts a summary of these metabolic pathways. Some studies have observed higher PHA yields with butyric acid (Carlozzi *et al.*, 2018), although Fradinho *et al.* noted that the presence of acetic acid accelerates the assimilation of propionic and butyric acid (Fradinho *et al.*, 2019). Also, the sole presence of acetic acid entails the accumulation of PHB only, and PHV accumulation is only possible in the presence of precursors such as propionic or valeric acid. Thus, propionate is converted to propionyl-CoA and condensates with acetyl-CoA to form PHV (Mukhopadhyay *et al.*, 2005). In addition, there are few studies on the production of polymers other than PHB or PHV with PPB, though C6 and C7 monomers (PHH among others) were detected in *Rhodospirillum rubrum sp.* (Brandl *et al.*, 1989). In general, the diversity of SCCA in the substrate increases the chances of PPB to accumulate longer-chain PHA, which is beneficial in the context of heterogeneous waste treatment. However, the presence of carbohydrates decreases PHA accumulation, shifting PPB metabolism towards photo-fermentative processes (Almeida *et al.*, 2021).

Other metabolic pathways that limit PHA accumulation are the diversion of electrons to H_2 production or the allocation of carbon to other storage products. During photofermentation, PPB evolves H_2 through nitrogenase

catalysis with adenosine triphosphate (ATP) consumption and electrons from reduced NADH. Nitrogenase activity is crucial for H_2 production by photosynthetic bacteria and is strongly inhibited by high ammonia concentration (Koku *et al.*, 2002). H_2 synthesis can occur in parallel with PHA accumulation on high COD and low ammonium substrates (Padovani *et al.*, 2016). **However, a more comprehensive investigation of this competition in heterogeneous substrates and mixed PPB cultures is still missing. Hence, a comprehensive examination of the mechanism of carbon allocation between different types of storage products such as glycogen and EPS has been addressed in this research**. Likewise, rather than eliminating the possibility of one route occurring by giving preference to others, synergies increase the value of bioconversion of PPB wastes into alternative energy sources and bioproducts. This information is crucial to better understand and optimize PHA production with PPB.
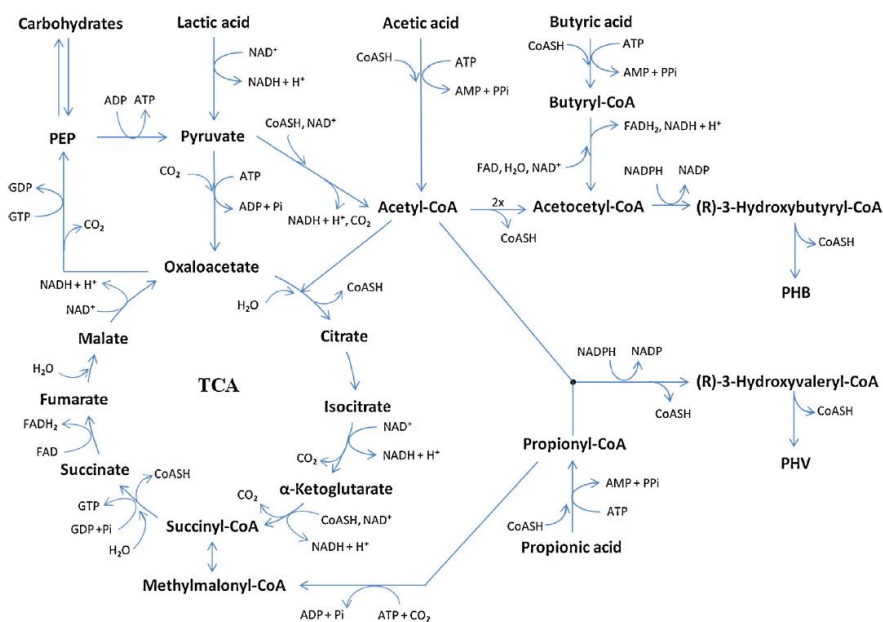

Figure 1.13. PPB assimilation and PHA accumulation pathways (Fradinho *et al.*, 2014).

1.6.4. PHA OPTIMIZATION WITH PPB

Although PHA accumulation with mixed PPB cultures has not been optimized, critical parameters like pH, irradiance, strain type, feed regime, and COD/nutrient ratio have been studied. Table 1.2. summarizes the studies found

on PHA production with mixed phototrophic cultures. Studies on PPB mixed cultures for PHA production are still limited, however, it can be observed the trend that, in recent years, waste has increasingly been studied as a substrate, replacing the use of synthetic substrates. **During the development of this Thesis, the use of OFMSW hydrolysates and fermentates for PPB growth and PHA accumulation was studied.**

Irradiance is one of the most important parameters in PPB culture, typically using monochromatic IR LED light sources. The optimal wavelength of 850 nm for COD and ammonia removal, as well as biomass growth, was determined on *Rhodopseudomonas sphaeroides sp.* (Qi *et al.*, 2017). However, this aspect is unexplored for PHA production. As for light intensity, it depends heavily on cell concentration. Light penetration controls the amount of light reaching the biomass. Higher cell growth reduces the light intensity reaching the cells and the overall productivity (Fradinho *et al.*, 2016). Mixed PPB cultures illuminated with a light intensity of 5.6-6.7 W g^{-1} (80 W m^{-2}) got the highest PHA content so far (60% on a dry basis) (Fradinho *et al.*, 2014). Thus, one of the major challenges in designing PPB accumulation bioreactors is the irradiance distribution at high biomass concentrations. Another lighting strategy studied is light/dark cycling to avoid photoinhibition problems (Montiel *et al.*, 2017).

PPB requires a higher proportion of nutrients than chemoheterotrophic systems, as their catabolic energy source arises from light, which directs a higher proportion of carbon to growth. The nutrients required can be related to the COD consumed, and a COD/N/P ratio of 100/7.1/1.8 is the ideal ratio for biomass growth in PPB (Puyol *et al.*, 2017). Presumably, values for PHA accumulation should be below this ratio. N limitation links to PHA accumulation (Sali and Mackey, 2021); however, a distinction between nitrogen sources used must be made. Ammonia availability is related to biomass growth due to nitrogenase inhibition, while the presence of sufficient glutamate as a nitrogen source enabled PHA accumulation in the environment (Carlozzi, *et al.*, 2019). Limitation of other nutrients such as P and S also allows PHA accumulation and is advantageous as that limitation does not imply production of H_2, a metabolic pathway that competes with PHA accumulation, as discussed above. An S-free environment links to higher PHA accumulation (Mukhopadhyay *et al.*, 2005). However, studies with P limitations have yielded opposite conclusions (Sali and Mackey, 2021), which may be due to the role of polyphosphate in PHA accumulation, a topic not yet addressed in the literature.

Table 1.2. Summary of studies of PHA production on PPB mixed cultures. Adapted from (Sali and Mackey, 2021).

Substrate	Light source	PHA (%wt)	PHA yield	PHA composition PHB: PHV	Refs
Acetic acid	Halogen lamps 16,000 lx	60%	0.45 g_{PHA} g_{COD}^{-1}	100:0	(Fradinho *et al.*, 2016)
Acetic acid	Halogen lamps 19,000 lx	20%	0.46 g_{PHA} g_{COD}^{-1}	100:0	(Fradinho *et al.*, 2013)
Propionic acid	Halogen lamps 19,000 lx	-	0.33 g_{PHA} g_{COD}^{-1}	51:49	(Fradinho *et al.*, 2014)
Acetic and butyric acid	LED light	44%	-	-	(Guerra-Blanco *et al.*, 2018)
Winery wastewater	LED light 4000 lx	-	0.001 g_{PHA} g_{COD}^{-1}	100:0	(Policastro *et al.*, 2020)
MSW fementate	Fluorescent lamps 4000 lx	-	0.55 g_{PHA} g_{COD}^{-1}	100:0	(Luongo *et al.*, 2017)
Acidogenic fermentation effluent	Fluorescent lamps 4000 lx	24%	0.212 g_{PHA} g_{COD}^{-1}	100:0	(Ghimire *et al.*, 2016)
Cheese whey	Halogen lamps 19,000 lx	20%	0.55 g_{PHA} g_{COD}^{-1}	88:12	(Fradinho *et al.*, 2019)
Fermented domestic wastewater	Halogen lamp 315 W.m^{-2}	31%	-	85:15	(Almeida *et al.*, 2021)

When working with mixed cultures, community analysis is an indispensable tool for optimizing bioprocesses. It is therefore essential to know which are the highest PHA-accumulating species. For example, *Rhodobacter sphaeroides* can accumulate up to 90% of PHA (Sangkharak and Prasertsan, 2007), whereas *Rhodobacter capsulatus*, *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* achieved accumulations of 24%, 45%, and 53%, respectively (Carlozzi, *et al.*, 2019; Hustede *et al.*, 1993). A lesser-known species, *Rubrivivax sp.*, accumulated 85% PHA (Ramana *et al.*, 2006). So far, with mixed PPB cultures, lower but encouraging values have been achieved, such as 60% using acetic acid as substrate (Fradinho *et al.*, 2016) or 31% using fermented domestic water (Almeida *et al.*, 2021). **Strategies to shift communities towards species capable of higher PHA accumulation are still a topic to be explored that has been studied in this research.**

Bioreactor designs could also affect the final output of PHA as light distribution and penetration, and biomass retention can be key factors for the cultivation system. A large surface area or surface-to-volume ratio is often preferred as it allows higher light distribution into the media. The most studied geometries include flat-panel, tubular, raceways and CSRT bioreactors:

a) Flat-sheet reactor offers a high surface-to-volume ratio and light exposure as it has a small thickness (Adessi and De Philippis, 2014), furthermore, In flat-panel bioreactors the membrane is easier to assemble into a membrane photobioreactor (MPBR) making volumes smaller and easier to scale up (Hülsen *et al.*, 2018). Hülsen *et al.* (2020) also explored a cylinder pipe-over flow-type photobioreactor to research to investigate the cultivation of PPB in suspended biomass and in biofilm, concluding that the latter is an interesting system to explore.

b) Tubular systems are tubes that can be arranged under different orientations such as vertical, horizontal, or serpentine. They provide high light penetration and avoid short-circuiting risks of flat-panel reactors, but have high energy requirements for pumping (Sirohi *et al.*, 2022). This type of reactor is mainly used for microalgae cultivation, although some studies have been done with PPB (Carlozzi *et al.*, 2019).

c) Raceway reactors are the most studied and applied in microalgae cultivation due to their low investment and operating costs (Sirohi *et al.*, 2022). However, they have many drawbacks such as control of temperature and water level, high chances of contamination etc. This type of reactor has been studied for the cultivation of PPB for protein production (Alloul *et al.*, 2021), and is currently being studied for the production of PHA.

d) CSTR-type photobioreactors are limited by the low area/volume ratio, which prevents uniform light distribution (Adessi and de Philippis, 2014). However, it provides easy scale-up, higher control of the process and better biomass mixing to ensure cells receive uniform exposure to light. Furthermore, the irradiation problem can be overcome through optical fibers or with IR-LED illuminator tubes that have been developed to overcome this disadvantage (Hülsen *et al.*, 2020).

A CSTR with internal illumination and a hollow fiber membrane was the type of bioreactor chosen for the continuous study of this Thesis. A novel MPBR configuration that has never been studied before.

1.7. INTEGRATION OF PHA INTO PHOTO-BIOREFINERY PROCESS

The global PHA production is expected to expand to meet the increasing market demand. However, the low PHA global productivity and overall process yield have limited the full-scale implementation of PHA production using microbial mixed cultures (MMC). The joint optimization of all the necessary steps (pretreatment, fermentation, accumulation) is key to determining the overall economic viability. Efforts on PHA production by MMC in the pilot-scale should focus on its integration with existing processes in waste plants in order to reduce production costs by exploiting the available infrastructure.

Several European projects are developing aerobic MMC projects for PHA production on a pilot scale. For example, the PHARIO project, developed in the Netherlands, has produced PHA for more than 10 months from activated sludge biomass and fermented streams rich in SCCA, resulting in high-quality PHA (Werker *et al.*, 2018). The RES-URBIS project developed a biorefinery in Treviso, Italy, where biogas and PHA were co-produced from the liquid fraction of OFMSW and WAS, achieving a yield of 7.6% PHA over the initial TS (Moretto, *et al.*, 2020). Other projects such as YPACK aim to optimize PHA applications for food packaging or VOLATILE the integration of PHA production in existing biogas plants. The performance of these pilot-scale plants is lower than those reported in laboratory-scale experiments, and further improvements are needed to foster the scale-up of the processes and reduce the overall production costs and environmental impacts.

Process integration becomes even more critical for energy integration, a key parameter in determining the technology's economic viability. In a study in the Lombardy region, pretreatment and separation of OFMSW into two phases (solid and liquid) for separated treatment for the production of PHA and biogas via AD produced a 30% higher energy balance than just AD (Papa *et al.*, 2022). Integrating PHA production into processes such as wastewater or MSW treatment plants, hydrogen production, or biodiesel plants could reduce production costs. In the future, the production of PHA from waste streams will require flexible processes, which could be adapted to different seasonal waste streams by adjusting operational variables. Various studies emphasize the potential and value of the double output in the context of industrial advantage presented by a stable, optimized simultaneous production system culminating from the bioprocesses aimed at a zero-waste strategy (Patel *et al.*, 2021; Thulasidharan *et al.*, 2021). However, scale-up studies of PHA production in PPB photobiorefineries are almost non-existent.

As discussed above, PPB has several advantages in producing PHA and has a metabolism capable of adapting to co-produce other high value-added products by adapting to the substrate and operating conditions seasonally. A recent life cycle assessment (LCA) analyzed the protein production with PPB from food waste (LaTurner *et al.*, 2020). This study inferred the importance of co-production with other high value-added products, e.g. with carotenoids. However, studies are still needed to determine the operating conditions that increase the PHA concentration and production rate by PPB using wastes. The production of PHA with PPB is still in its infancy, but it has entered a new phase. For example, the INCOVER proyect (https://incover-project.eu/) presents research solutions to recover energy (biomethane) and bioproducts (bioplastics, organic acids, biofertiliser, biochar, irrigation water) from municipal, industrial and agricultural wastewater using PPB. Another relevant project is DEEP PURPLE (https://www.deep-purple.eu/) which is establishing the first pilot-scale PPB-based photobiorefinery in Europe. The DEEP PURPLE concept relies upon a versatile, integrated and flexible Multi-Platform Biorefinery, based on the metabolism of PPB to extract and recover high added-value compounds such as PHA, ectoine and cellulose from urban waste (e.g. OFMSW). **The results obtained in this Thesis have provided the foundation for the development of the production of PHA in the DEEP PURPLE project.**

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CHAPTER 2: INITIAL HYPOTHESIS AND RESEARCH OBJECTIVES

2.1.   CONTEXT AND RESEARCH GAP

This Doctoral Thesis focuses on the valorization of the OFMSW by coupling thermal and biological treatments into bioplastics (PHA) and other high-value-added products. It applies the concept of a circular economy for the holistic treatment of this waste, tackling two problems simultaneously, the OFMSW management and disposal concerns and the environmental burden posed by petroleum-based plastics. The key biological treatments are anaerobic digestion, acidogenic fermentation, and photoheterotrophic process with PPB, the latter being the keystone of the Thesis. Hence, the main objective of the research was to design a photobiorefinery using that keystone as its core and optimize PHA accumulation. Before this Thesis, PPB growth on OFMSW derivates had never been studied, nor the preliminary design of a PPB-based photobiorefinery for the OFMSW valorization had been attempted. Therefore, the following questions have arisen:

1)   Is it possible to grow a mixed culture of PPB and accumulate PHA using OFMSW hydrolysates?

2)   How does using a carbohydrate-based residue such as lignocellulosic waste affect the PHA accumulation by PPB?

3)   How do high percentages of lignocellulosic residue affect the acidogenic co-fermentation of the OFMSW?

4)   Is it possible, and which parameters are key to maintain a good PHA productivity from OFMSW fermentate?

5)   Is the anaerobic digestion process suitable to close the biorefinery's carbon cycle and energy balance?

6)   What are the major challenges in scaling up this technology?

With the purpose to answer these questions, the discussion Chapter of the Thesis was organized in four different sections with the following objectives:

Section I: A preliminary proof of concept was designed consisting of thermal pretreatment of the OFMSW coupling with an anaerobic digestion process of the solid fraction and the growth and accumulation of PHA by mixed cultures of PPB from the liquid fraction in batch tests. OFMSW and lignocellulosic wastes were used as feedstock. In addition, preliminary mass and energy balances of the process were accomplished to check the energetic sustainability of the process.

Section II: The acidogenic co-fermentation of food waste (FW) and lignocellulosic waste (LW) was performed. Due to the lack of knowledge on how an increase in lignocellulosic waste can affect the acidogenic fermentation process, several mixtures were studied. Synergies in the SCCA and H_2 production and the effect of a pilot-scale steam explosion pretreatment on the process were analyzed for the first time.

Section III: A conceptual design of a photobiorefinery was studied based on the integration of a photoheterotrophic process in an MPBR with mixed cultures of PPB and fed with the liquid effluent coming from the acidogenic fermentation. The initial feedstock was thermally pretreated in a steam explosion reactor before the fermentation step. PHA production optimization was addressed with a special focus on the effect of microbial communities as well as carbon and electron allocation. This section finalizes with a conceptual flow diagram of the overall process including preliminary mass and energy balances.

Section IV: A critical literature review was carried out to identify possible strategies for the industrial scale-up of the photoheterotrophic process with PPB. Key parameters were analyzed as well as possible strategies used in other technologies to understand the prospects of the project.

The following scheme shows the working plan with the integration of the aboved-mention sections.

2.2.   WORKING PLAN
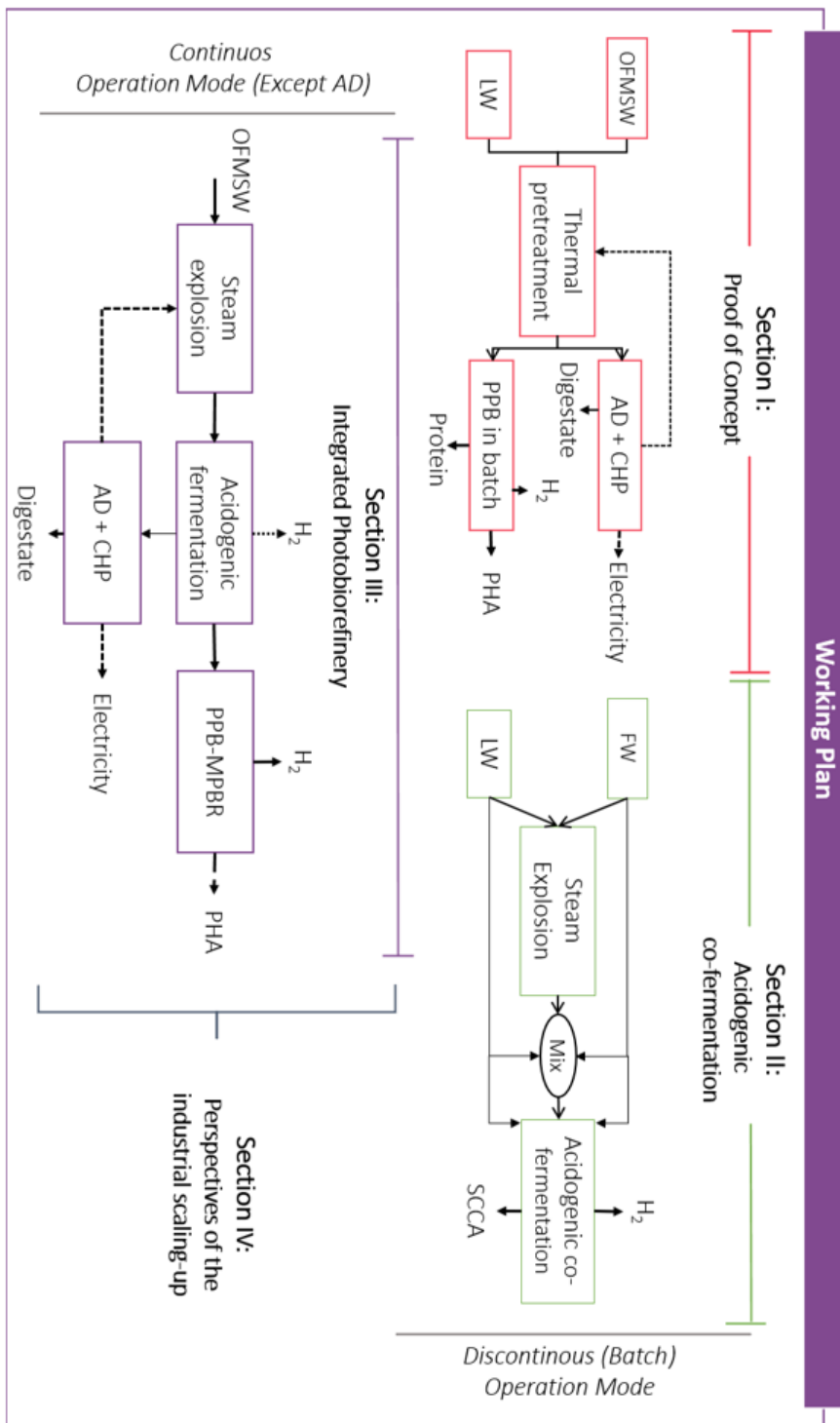
CHAPTER 3: MATERIALS AND METHODS

3.1. MATERIALS AND PROCESSES

3.1.1. WASTE SOURCES

Different sources of waste were used throughout the Ph.D. Thesis.

1) OFMSW. In section I, two different OFMSW were selected based on their volumetric importance in real-scale plants. Samples were collected from an urban waste treatment facility located in Madrid (Spain), one immediately after fresh dumping from vehicles coming from a selective organic fraction (Pre-Sorted by the citizens), and the other one previously sorted by the waste treatment plant (Selective). Different macroscopic parameters were obtained in the OFMSW used in section 3, showing the significant heterogeneity of this waste.

2) FW and LW were used In section II. The origin of the FW samples is the Mercamadrid food wholesale market, composed mainly of fruit and vegetables. LW samples correspond mostly to prune and gardening residues. Both were collected from a solid waste plant in Madrid (Spain).

The waste was always blended and homogenized with an electric mixer grinder to achieve less than 10 mm particle size and then stored at 4 °C until further use. Table 3.1 summarizes the main macroscopic characteristics of the organic wastes used as feedstock during the Ph.D. Thesis.

Table 3.1. Average values with 95% confidence intervals for the macroscopic characteristics of the organic solid waste used.

	Section 1		Section 2	Section 1 and 2	Section 3
Waste	OFMSW Pre-sorted	OFMSW Selective	FW	LW	OFMSW
TS (g kg^{-1})	367 ± 51	343 ± 42	115 ± 12	950 ± 12	185 ± 21
VS (g kg^{-1})	298 ± 46	297 ± 19	99 ± 6	911 ± 11	159 ± 13
TKN (gN kgTS^{-1})	3.7 ± 1.7	3.5 ± 0.8	3.2 ± 0.5	2.5 ± 0.8	2.9 ± 0.3
TCOD (g L^{-1})	143.9 ± 12.5	135.5 ± 6.7	122 ± 5	1075 ± 8*	185 ± 10
SCOD (g L^{-1})	11.2 ± 0.2	10.5 ± 0.2	9.5 ± 0.6	-	2.3 ± 1.1

*g gTS^{-1}

3.1.2. GROWTH MEDIA

In this Ph.D. Thesis, two synthetic cultivation media were used for inoculum adaptation and control tests when necessary. The carbon source used was:

1) Acidogenic Fermentation Medium (AFM): For the acidogenic fermentation, a complex synthetic feed of starch, sucrose, peptone, and frying oil in a ratio of 1:1:1:0.1 g kg^{-1} was used as a carbon source.

2) Modified Ormerod Medium (MOM): For PPB photoheterotrophic tests, a synthetic substrate mixture of HAc:HPr:HBu:EtOH on a 1:1:1:1 COD basis (2 gCOD L^{-1} in total) was used as a carbon source.

The culture's composition was adapted from Ormerod *et al.* (1961), as follows: Macro nutrients (g L^{-1}): 1.086 K$_2$HPO$_4$·3H$_2$O; 0.666 K$_2$HPO$_4$; 0.4 NH$_4$Cl; 0.075 CaCl$_2$·2H$_2$O; 0.2 MgSO$_4$·7H$_2$O and 0.007 FeCl$_2$ and 0.02 of yeast extract. Micro nutrients (mg L^{-1}): 2.81 H$_3$BO$_3$; 2.02, 2.05 CoCl$_2$; 2.02 MnCl$_2$·4H$_2$O; 0.16 Na$_2$SeO$_3$·5H$_2$O; 0.09 NiCl$_2$; 0.55 (NH)$_6$Mo$_7$O$_{24}$·4H$_2$O; 0.114 ZnCl$_2$; 0.028 CuCl$_2$·2H$_2$O; 0.015 Biotin and 2.01 EDTA.

3.1.3. INOCULA

Different types of inocula were used during this Thesis. They are summarised in three main sources of inocula:

1) For the anaerobic digestion process, a fresh methanogenic anaerobic inoculum was obtained from the urban wastewater treatment plant placed in Mostoles (Madrid).

2) For the acidogenic fermentation process (batch and continuous tests), the methanogenic sludge was inoculated into an anaerobic fermenter and maintained at 55 °C and 5.5 pH to avoid methanogenic activity. This reactor was fed with AFM and operated for more than 2 years, resulting in a highly acclimatized anaerobic acidogenic thermophilic sludge that produced butyrate predominantly (40% of total SCCA). Figure 3.1 shows the SCCA composition of the effluent obtained from this reactor.
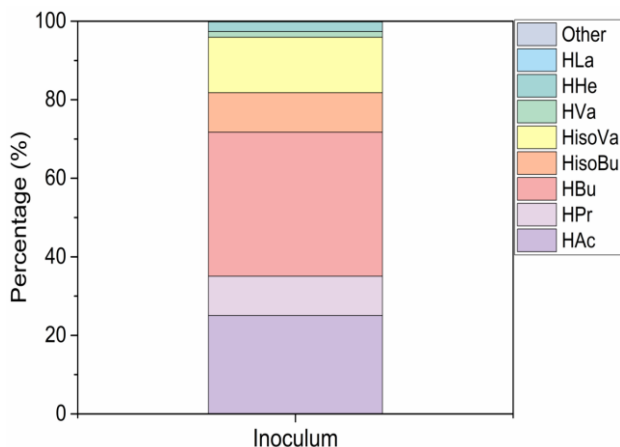
Figure 3.1 SCCA composition of the effluent obtained during continuous incubation in an anaerobic fermenter with an inoculum for non-pretreated waste. Hac: Acetic acid, HPr: Propanoic Acid, HBu: Butiric acid, HisoBut: Isobutiric acid, HisoVa: Isovaleric acid, HVa: Valeric acid, HHe, Hexanoic acid and HLa: Lactic acid

3) For the phoheterototrophic process (both batch and continuous processes), the active biomass of the mixed culture of PPB was obtained from an MPBR described elsewhere (de las Heras *et al.*, 2020). This inoculum was fed with the MOM and grown in batch culture for almost three years in an incubator illuminated with IR lamps (Philips, BR125 IR, España) at around 45 W m^{-2} and covered with a UV/VIS filtering foil. Every week, the culture media was refreshed (90% of the volume).

3.1.4. THERMAL PRETREATMENTS

Two thermal hydrolysis reactors were used: A laboratory-scale autoclave reactor and a pilot-scale steam explosion reactor. Each of these reactors is detailed below:

1) Section I. Hydrothermal pretreatments for the proof of concept were performed in a 1-L autoclave (Parker, Autoclave Engineers, USA) at 150 rpm with a mass solid-to-liquid ratio of 1:5 and under autogenous pressure conditions (Westerholm *et al.*, 2019). The autoclave was heated to the target temperature with an average heating rate of 6 °C min^{-1}. Upon the treatment, the samples were cooled with ice until room temperature to minimize the volatilization of organic compounds. After that, the samples were centrifuged at 6000 rpm for 10 min to separate solid and liquid phases. Both fractions were stored at 4 °C until further use.

2)   Section II and III. The pilot-scale steam explosion reactor consists of a steam boiler (Certuss E56, Krefeld, Germany) and a 20-L total volume hydrolysis reactor (working volume of 10 L) connected to a flash tank (100 L). The reactor load was 6 kg of waste per batch. Steam input at 10 bar achieved a heating rate of 18±4 °C min^{-1} up to the target temperature. Upon the reaction, a sudden opening of the reactor caused a steam explosion in the flash tank, and the treated wastes were extracted and cooled to ambient temperature. The hydrolysis reaction was set at 150 °C for 40 min. These parameters were selected based on an optimization carried out by our research group within the framework of the Deep Purple project, but whose data are not shown in this Ph.D Thesis.

Water was added to LW to reach 20% TS, to match it to FW or OFMSW. The phases were separated by centrifugation at 6000 rpm and 10 min after the hydrolysis reaction if needed. The complete hydrolysate was stored at 4 °C until further use if not needed.

3.1.5. Thermophilic acidogenic fermentation

Batch experiments (Section II). Each biochemical hydrogen potential (BHP) test was performed in sextuplicate, allowing a fermentation period of 8 d. One triplicate served for measuring daily biogas production at a constant liquid level, while a second triplicate was used to measure daily COD dynamics. Experiments were conducted in a 160 mL Pyrex flask. A thermostatic incubator maintained a temperature of 55 °C and kept the bottles in constant shaking during the reaction time. The addition of 1 M HCl and KOH solutions allowed the setting of the pH at 5.5 for all conditions.

To correctly adjust the inoculum-substrate ratio, preliminary tests were performed with both untreated and pretreated substrates (Figure 3.2). The inoculum was added at 5% by volume to prevent a high initial SCCA presence that could falsify the results. Different substrate-to-biomass loadings (gCOD gVSS^{-1}) were tested, and conditions were chosen based on hydrogen production as it is a fast and easy-to-measure response indicator. Its high output is related to the metabolic pathways of acetic and butyric acid formation, which is widely reported (Hoelzle *et al.*, 2014). Consequently, a ratio of 25 gCOD gVSS^{-1} was chosen for the untreated wastes and 5 gCOD gVSS^{-1} for the pretreated ones. The bottles were filled to a volume of 120 mL with milli-Q water. Negative control tests were also performed using both the inoculum

alone (considering the endogenous production of hydrogen and SCCA) and the substrates without inoculum (eliminating interferences in the estimations).
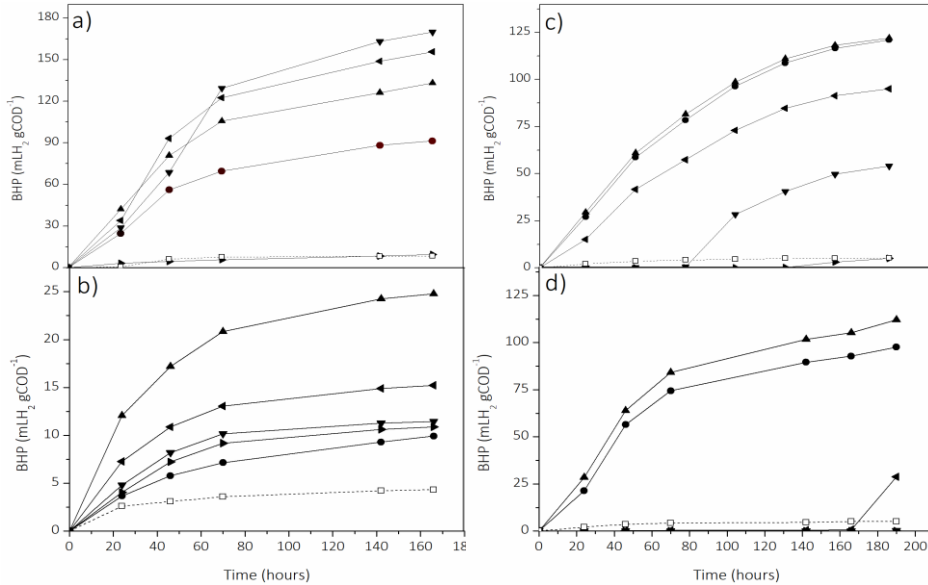

Figure 3.2 Hydrogen potential in preliminary tests (BHP) at the different FW (a), LW (b) and pretreated FW (c) and LW (d) concentrations (gCOD gVSS^{-1}). On all panels □ represents the inoculum while ●, ▲, ▼, ◄, ► represents 10, 25, 40 75 and 120 (gCOD gVSS^{-1}) on panels (a) and (b) and 2, 5, 10, 20 and 30 (gCOD gVSS^{-1}) on panels (c) and (d), respectively.

Before conducting the experiments, all the elements used, such as the flask bottles, tubes, substrates, or milli-Q water, were heated up to 55°C to avoid a lag phase. The bottles were sealed with rubber stoppers and flushed for 5 min with Argon to remove any residual oxygen. Liquid samples were extracted from one triplet of bottles and filtered through a cellulose-ester filter of 0.45 μm of pore size (Advantech) to monitor the pH evolution and determine the SCOD and the SCCA composition of the fermentation broth. Gas samples were extracted from the other triplet to analyze the H$_2$ production and gas composition.

Continuous experiments (Section III). This process was carried out in an acidogenic CSTR reactor with a total volume of 2.5 L (working volume of 2 L), as shown in Figure 3.3. The thermophilic temperature (55 °C) was controlled with an external water bath connected to an external jacket of the reactor. The HRT was fixed at 5 d, and the pH was set at 5.5 with a PLC. One outlet on the

headspace was connected to a flowmeter (Ritter, Germany), while the other was sealed with a rubber stopper to take biogas samples with a gas syringe. The anaerobic state of the reaction was ensured both in the reactor and in the feed and outlet bottle. Anaerobic conditions were established in the substrate by sparging the medium with Ar and reducing it with $Na_2S + 9H_2O$ (1 mL per liter). No more than 0.5% O_2 was observed in the reactor headspace during the experimental period.
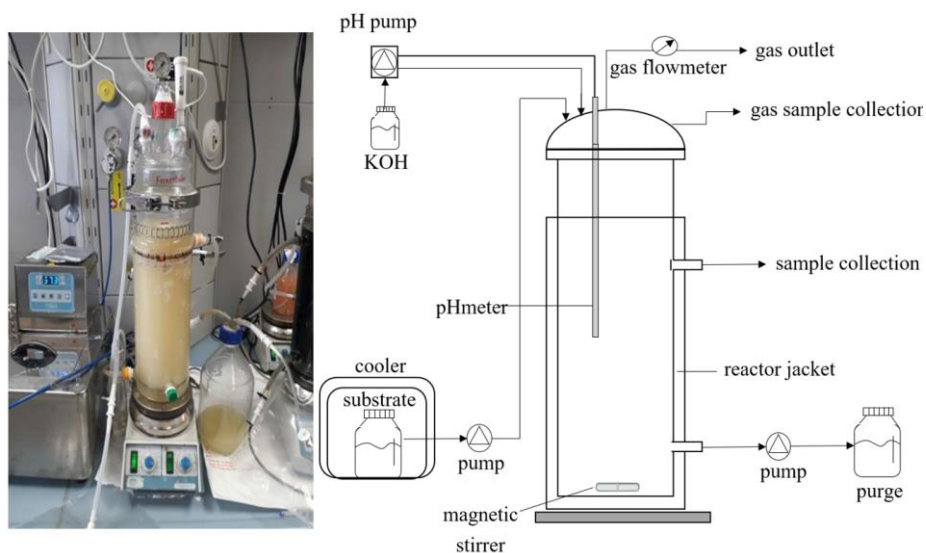
Figure 3.3 Picture (left) and schematic representation (right) of the continuously-fed thermophilic acidogenic fermentation reactor.

3.1.6. ANAEROBIC DIGESTION PROCESS

All anaerobic digestion processes studied were carried out through standard biochemical methane potential (BMP) tests. The tests were performed by triplicate, yielding $mLCH_4$ gVS^{-1}, in 160 mL serum bottles at mesophilic conditions (37 ± 0.5 °C) following Angelidaki *et al.* (2009). The organic substrate used was the solid fraction after centrifugation of the hydrolysate. An inoculum to substrate (I/S) ratio of 2:1 (as VS) and an initial concentration of 10 gVS L^{-1} were set up. A triplicate control of the inoculum was used to subtract the methane production coming from its endogenous digestion.

3.1.7. PPB PHOTOHETEROTROPHIC PROCESS

Batch tests (Section I). The activity of the phototrophic biomass was determined by Specific Phototrophic Activity (SPA) batch tests following the indications in Hülsen *et al.* (2016). Triplicate experiments were performed in 160 mL anaerobic serum bottles in a temperature-controlled incubator (at 30 ^{0}C) with an initial pH of 6.5. The incubator was illuminated with IR lamps (Philips, BR125 IR, España) at around 45 W m^{-2} and covered with a UV/VIS filtering foil. An inoculum was added at 10 mg VSS L^{-1}, and the organic substrate (the liquid fraction obtained after centrifugation of the hydrolysate pretreated through thermal hydrolysis) was diluted with Milli-Q water to a concentration of 1 gCOD L^{-1}. Control tests fed with the MOM described before were used to compare with the experimental tests.

The light absorption spectra of the culture over the visible and near-infrared range (VIS-NIR) it was checked to verify that the biomass has two prominent adsorption peaks at 805 and 865 nm, which corresponds to the absorption maxima for the bacteriochlorophyll *a*, indicating an evident enrichment in PPB (Hülsen *et al.*, 2016). Biomass samples were also extracted at the end of the experiments and fixed with formaldehyde at 0.2% volume for 1 h and 4°C to measure PHA content, thus calculating the PHA production yield.

Continuous tests (Section III). This process was carried out in a MPBR inoculated with a mixed culture of PPB. Figure 3.4 shows the scheme of the MPBR. A 2.5-L reactor (working volume of 2 L) with a submerged LED lamp emitting at 805nm (Idea Bioprocess technology, Italy) that provides a volumetric irradiance of 2.2 W L^{-1}. A submerged hollow fiber membrane (Zena s.r.o, Czech Republic) was also used to discharge the filtered output. A pH-meter with a control system was used to maintain the pH above 6.5 by dosing KOH (0.1 M). The reactor head had two gas outlets, one sealed with a rubber stopper dedicated to taking biogas samples and the other connected to a flowmeter (Ritter, Germany) to measure the volume of gas produced. The MPBR head was opened every other day to clean the submerged LED lamp and to avoid biofilm formation. Each time this happened, the reactor headspace was purged with Ar to ensure anaerobic conditions. As in the acidogenic fermentation process, the anaerobic state of the reaction was ensured in the reactor and the feed and outlet bottle. The feed bottle was kept in a cooler at 4 ^{o}C.
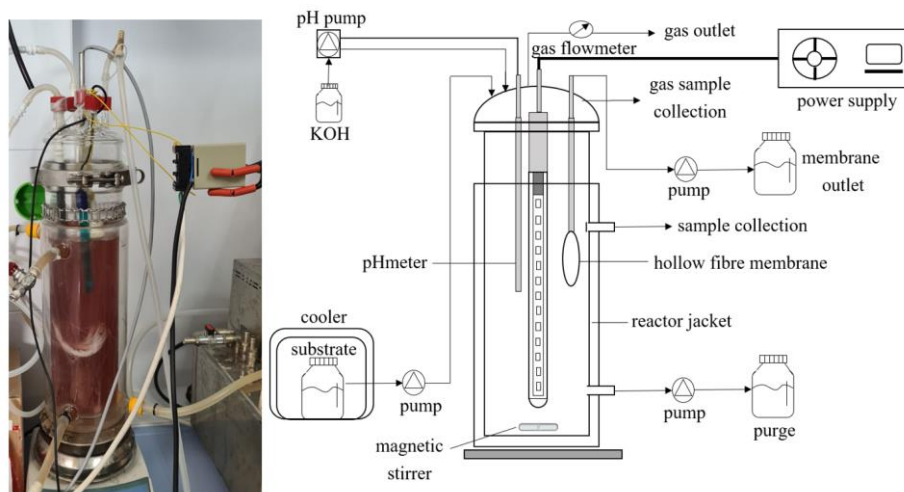
Figure 3.4 Picture (left) and schematic representation (right) of the MPBR.

3.2. METHODS

3.2.1. ANALYTICAL METHODS

Analytical determination of pH, total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), total Kjeldahl Nitrogen (TKN), total phosphorus (TP) and COD (TCOD and SCOD) are carried out following Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Ammonia (NH_4^+), and phosphate (PO_4^{3-}) concentrations dissolved in the aqueous phase are determined using Smartchem 140 (AMS Alliance), following APHA-AWWA Standard Method 4500-NO2 B and 4500-P E, respectively (APHA, 2005). Liquid samples are filtered through a cellulose-ester filter of 0.45 μm of pore size prior to the analysis (Advantech, Japan). Elemental analysis (C, H, N, and S) is performed by an elemental analyzer (Vario EL III, Elemental Analysis System GMHB, Germany). SCCA and monosaccharides are analyzed using an ion-exclusion RazexTM ROA-Organic Acid H+ HPLC column (Phenomenex, USA), coupled to a refractive index detector (Agilent, USA) and operated at 65 °C and 1 mL min^{-1}, with 0.005 M H_2SO_4 as the eluent.

Gas pressure is measured using a Boyle-Mariotte apparatus. The composition of each reactor's headspace is analyzed using a 7820A gas chromatography (GC-TCD) system (Agilent Technologies, Santa Clara, CA, USA). The mobile phase is Argon at a 5 mL min^{-1} flow rate. The temperature of the oven and the detectors are 45 °C and 220 °C, respectively.

The metal content is determined using inductively coupled plasma-optical emission spectrometry ICP-OES (Varian Vista AX Pro, USA). First, 0.5 g of the sample is measured, and 10 mL of Nitric acid and 4 mL of Hydrochloric acid are added. Then it is digested for 2 h at 175 °C and diluted at 1:10 before being measured. The determination of the concentration of the metals involves the creation of calibration curves corresponding to each metal, with six calibration points for each metal. The solutions are prepared from certified standard solutions for atomic emission analysis of 1000 mg L^{-1} in nitric acid medium. The correlation coefficient r^2 obtained for all cases are 0.999.

The effect of thermal pretreatment over solid samples is evaluated through the characterization of the samples using mid-infrared spectroscopic (FTIR) and X-ray diffraction (XRD) tests. Firstly, the samples are dried at 100 °C in an oven. For XRD, samples as flat as possible are correctly distributed on a glass holder that does not produce diffraction effects. A X'Pert PRO diffractometer (Malvern Panalytical, Netherlands), with θ / 2θ geometry, is used, using Cu-Kα radiation. The data are collected from 5 to 90º (2θ) with a resolution of 0.02º.

The crystallinity index is calculated according to Eq. [3.1]:

$$CrI = \frac{I_{002} - I_{amorphous}}{I_{002}} \; x \; 100 \qquad \text{Eq. [3.1]}$$

Where CrI is the crystallinity index, I$_{002}$ is the maximum intensity of the 002 peaks at 2θ = 22.5°, and I$_{amorphous}$ is the intensity at 2θ = 18.7°

The FTIR spectra are recorded between 400 and 4000 cm^1 using a Spectrum 100 system (Perkin Elmer, US). Samples are prepared by grinding the solid fractions on an agate mortar and pressed at 10 MPa.

For PHA content quantification, biomass samples are collected, and 0.2% (by vol.) formaldehyde is added to stop the microbial activity. The samples are then lyophilized overnight to remove water. Commercial standards of PHA are used to make calibration curves: one containing 88% of hydroxybutyrate (HB) and 12% of hydroxyvalerate (HV) and another with 100% Methyl 3-hydroxyhexanoate (Sigma-Aldrich, USA). Dried biomass samples of 1–2 mg are weighted using an analytical balance (Radwag, Poland) and transferred into round base glass tubes. Volumes of 2 mL of ultra-pure trichloromethane (CHCl$_3$) and 2 mL of a standard solution (97% methanol, 3% H$_2$SO$_4$, and 200 mg L^{-1} sodium benzoate) are added. Tubes are heated for 20 h at 100 °C and then

cooled at room temperature. 3 mL of milliQ water is added to each sample, and two phases (aqueous and organic) are formed. The organic phase is analyzed in a gas chromatograph coupled to a Flame Ionization Detector (GC-FID Varian CP-3800). When an unidentified peak is discovered in the GC-FID, a check is done using a 320-MS GC Triple Quadrupole Mass Spectrometer with a Rxi-5Sil Colum to identify this peak.

The method used for EPS quantification is adapted from Felz *et al.* (2016). For each measurement, 1 L of biomass culture is collected from the reactor outlet. The biomass is centrifuged at 4000 x g for 20 min, and the supernatant was removed. The biomass pellets are collected, and the TS and VS are measured. The formaldehyde extraction method is used. First, at least 1 g (wet weight) of biomass is measured in an ISO bottle and filled to 50 ml with demineralized water. Then, 0.3 ml of 37% formaldehyde is added, and the bottle was shaken and left in a refrigerator at 4°C for one h. Subsequently, 20 mL 1M NaOH is added, and the bottle is shaken again and kept in a refrigerator for 3 h. Once finished, the sample is centrifuged at 4000 x g for 20 min, and the supernatant is collected. This supernatant is dialyzed (3500 Da) for 24 h in 1 liter of ultrapure water. Finally, TS and VS are measured to the dialyzed supernatant to calculate the dry weight of the EPS.

The glycogen measurement is based on a method adapted from Lanham *et al.* (2012). Briefly, after overnight lyophilization, 1 to 2 mg of dry biomass are weighed, then digested with 2 ml of 0.9M HCl and dissolved in a thermoblock for 3 h at 100°C. Subsequently, samples are quickly cooled on ice and filtered with a 0.45 cellulose-ester filter. Finally, glycogen is quantified by HPLC measurement using the same method described for SCCA quantification. The calibration curve is performed with standard glycogen from oysters (Sigma-Aldrich, USA).

3.2.2. BIOLOGICAL METHODS

Total DNA is extracted with a Microbial DNA isolation kit (Soil DNA Isolation Kit, CANVAX, Cordoba, Spain) to perform the molecular identification of prokaryotes and frozen to -20 °C for 4 d until use. The rest of the analysis is carried out by the FISABIO (Valencia) Sequencing and Bioinformatics Service research center. A brief summary of the protocol used is described below:

The quantification, amplification (PCR) and 16S rRNA gene measurements, and the amplicon taxonomic annotation and comparative analysis are outsourced to Instituto ISABIAL-FISABIO, Hospital General Universitario de Alicante, Alicante, Spain. Single molecule real time sequencing sequencing is performed on a PacBio RS II instrument. The preparation of the library is carried out through the 27F: AGRGTTYGATYMTGGCTCAG and 1492R: RGYTACCTTGTTACGACTT universal primer set to amplify the full-length 16S rRNA gene from the genomic DNA. Both the forward and reverse 16S primers are tailed with sample-specific PacBio barcode sequences to allow for multiplexed sequencing. Those barcoded primers are chosen because this reduces chimera formation compared to the alternative protocol in which primers are added in a second PCR reaction. The KAPA HiFi Hot Start DNA Polymerase (KAPA Biosystems) is used to perform 23 cycles of PCR amplification, denaturing at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 60 s. Post-amplification quality control is performed on a Fragment analyzer (Agilent Technologies, Santa Clara, CA, USA). Amplified DNA from the samples is then pooled in equimolar concentration before loading.

Bioinformatics analysis is performed employing Pacific Bioscience system. Data are demultiplexed, and Circular Consensus Sequences (CCS) are called using the SMRT-Link analysis software (v9). The obtained CCS are submitted to the quality check pipeline following the DADA2 R Statistics package (v1.20.0) (Callahan *et al.*, 2016), which reorients the sequences, removes forward (27F, AGRGTTYGATYMTGGCTCAG), and reverse (1492R, RGYTACCTTGTTACGACTT) primers, filters and trims the sequences by length and average quality, dereplicates and estimates sequencing errors using PacBio error's model, and infers the compositions of the samples. The non-redundant sequences data set and the features abundance table are obtained using the previous steps entered the QIIME2 pipeline (v2021.4) (Bolyen *et al.*, 2019). Sequences that do not match any 16S rRNA are filtered out using the VSEARCH algorithm (Rognes *et al.*, 2016) against the Silva138 reference. Features of less than ten reads among all samples are also filtered out. The remaining sequences are annotated using the sklearn algorithm against the Silva138 QIIME2 classifier reference database (Quast *et al.*, 2013) (clustered at 99% of similarity). The taxonomic annotations are then used to compile contingency tables at every taxonomic rank.

3.3. CALCULATIONS

3.3.1. DATA HANDLING AND STATISTICAL ANALYSIS

Unless otherwise stated, error bars and ± symbols are always 95% confidence intervals. Statistical analyses, modeling equations, and performance parameters used in each of the technologies in this Thesis are detailed below.

a) Performance parameters of thermal hydrolysis pretreatment

VS destruction in the thermal hydrolysis was expressed according to Eq. [3.2]:

$$VS\ destruction\ \% = \frac{(VS_{in} - VS_{out})}{VS_{in}}\ x\ 100 \qquad \text{Eq. [3.2]}$$

, where VS_{in} and VS_{out} are the VS concentration before and after the thermal hydrolysis in the solid, respectively. For steam explosion, the steam entering the hydrolysis reactor is considered the same as the steam leaving the flash tank. The organic matter and nutrients recovery in the liquid fraction (solubilization of COD, N, and P) was calculated according to Eq. [3.3]:

$$Solubilization\ \% = \frac{(C_{To} - C_{Sol})}{C_{To}}\ x\ 100 \qquad \text{Eq. [3.3]}$$

, where C_{To} and C_{Sol} are the COD, N, or P concentrations (dry basis) on the initial raw waste and the remaining solid fraction after centrifugation, respectively.

The obtaining of comparably and reliably effects of hydrolysis on the treatment of solids due to time and temperature is usually carried out through the calculation of the severity factor, log R_0 (Overend and Chornet, 1987). This factor, though subjected to some limitations, enables a more straightforward comparison among different tested conditions (Eq. [3.4]).

$$Ro = \int_0^t exp\left(\frac{T(t) - T_{Ref}}{14.75}\right) dt \qquad \text{Eq. [3.4]}$$

In this instance, *t* is heating time (min), *T* is the target temperature (°C), T_{Ref} is the reference temperature of 100 °C, and 14.75 is based on the activation energy when assuming pseudo-first-order kinetics.

b) *Performance parameters of acidogenic fermentation*

To better understand the results of acidogenic fermentation, we use several parameters like the extent of hydrolysis (H_E), which delimits the overall efficiency of acidogenic fermentation. The acidification extent determines the percentage of SCCA (as COD) compared to the released SCOD. Both are calculated according to Eq. [3.5] and [3.6]:

$$H_E = \frac{COD_{H_2} + SCODf - SCODi}{TCOD - SCODi} \times 100 \qquad \text{Eq. [3.5]}$$

$$Acidification\ extent = \frac{COD_{SCCA}}{SCOD_f} \times 100 \qquad \text{Eq. [3.6]}$$

, where COD_{H2} represents the amount of COD converted into hydrogen gas, expressed as COD equivalents, considering the ratio of 8 gCOD gH$_2^{-1}$. Total SCCA production (COD_{SCCA}) is the sum of each SCCA.

The conversion yield of the total COD added from the substrate (TCOD) to the SCCA in the fermentation process is determined as the SCCA yield (Y_{SCCA}), according to Eq. [3.7]:

$$Y_{SCCA} = \frac{COD_{SCCA}}{tCOD} \times 100 \qquad \text{Eq. [3.7]}$$

Finally, the synergistic effect (φ) produced by the co-fermentation of FW and LW can be determined mathematically by dividing the measured variable by the calculated theoretical variable according to Eq. [3.8]:

$$\varphi = \frac{He_i}{(He_{FW} \times \alpha_i + He_{LW} \times \beta_i)} \qquad \text{Eq. [3.8]}$$

, where the individual H_E of each waste (H_{EFW} and H_{ELW}) are multiplied by each mixing ratio from the co-fermentation mixture (α and β). Obviously, φ values over 1 would mean a presence of a synergistic effect.

c) *Performance parameters of photoheterotrophic process*

Microbial protein estimation is calculated based on NH$_4$-N and TKN content (solid TKN x 6.25) following Eding *et al.* (2006), according to Eq. [3.9]:

$$Protein\ (mgN\ L^{-1}) = (TKN_t - TKN_s) * 6.25 \qquad \text{Eq. [3.9]}$$

, where TKN$_t$ is the TKN from the culture broth and TKN$_s$ is the TKN from the soluble fraction (after filtration).

The percentage of intracellular/extracellular compounds accumulated (Y_{PHA}, Y_{GLY}, Y_{EPS}) is expressed as dry weight (wt %) and calculated according to Eq. [3.10]:

$$Accumulation\ (wt\ \%) = \frac{M}{VSS} * 100$$

Eq. [3.10]

, where M is the measured mass of PHA, Glycogen or EPS (g), and VSS is the dry mass of Volatile Suspended Solids in the medium (g).

For the statistical analysis of the evolution of the bacterial composition determined by PacBIO, a principal component analysis (PCA) is performed to explore the variation of the communities over time. Distance-based redundancy (RDA) is conducted to reveal potential associations and multivariate relationships between environmental factors (OLR, sludge retention time (SRT), PHA, Glycogen, VSS, and hydrogen production) and the relative abundance of phylotypes at the genre level. Both PCA and RDA are carried out using the "Vegan" Package in RStudio (R Development Core Team). Relationships between samples, bacterial communities, and environmental parameters are examined using multiple linear regression analysis to rule out linear variables (>95%).

3.3.2. MODELING

a) Modeling and statistical analysis of BMP

Kinetic parameters are obtained by fitting the data to a first-order model simplified from the IWA Anaerobic Digestion Model no. 1 (ADM1) (Batstone *et al.*, 2002), thereby calculating the first-order hydrolysis constant k_H (d^{-1}) and the biodegradability, B_O (mLCH$_4$ gVS^{-1}). The following equations describe the model (Eqs. [3.11]-[3.12]):

$$\frac{dS}{dt} = -k_h * S$$

Eq. [3.11]

$$\frac{dB}{dt} = \frac{-B_o * dS}{dt} = k_h * B_o * S$$

Eq. [3.12]

, where k_H: First-order constant (d^{-1}), S: Biodegradable substrate (kgVS L^{-1}), B methane production (LCH$_4$ kgVS^{-1}), B_o: B at infinite time (LCH$_4$ kgVS^{-1}) and t: time (d).

Statistical significance is analyzed by calculating the confidence intervals (at 95%) for all the experimental data and the estimated parameters, when possible. Parameter uncertainty surface is obtained as previously described (Batstone *et al.*, 2003), and confidence intervals are also calculated based on two-tailed t-tests from parameter standard error for statistical representative comparison. All parameter uncertainty analyses are performed using Matlab.

b) Modeling of the photoheterotrophic process

Apparent biomass yield ($Y_{x/s}$ in gVSS gCOD^{-1}) is calculated as the ratio between the total biomass growth measured as VSS and the total consumed COD according to Eq. [3.13].

$$Y_{X/S} = \frac{gVSS_{biomass}}{gCOD_{consumed}}$$

Eq. [3.13]

The specific substrate rate, k_M (gCOD gVSS^{-1} h^{-1}) is estimated by fitting the experimental data to the differential equation specified in Eq. [3.14] by using the software Aquasim 2.1d, as in Puyol *et al.* (2017).

$$\frac{dS}{dt} = k_M * X_{ph} * \frac{S}{K_s + S}$$

Eq. [3.14]

, where S is the concentration of soluble substrate (mgCOD L^{-1}), K_s is the saturation constant for S consumption (mgCOD L^{-1}), X_{ph} is the concentration of photoheterotrophic biomass (mgVSS L^{-1}), and t is time (d).

The specific production rates of H$_2$ (q_{H2}, mlH$_2$ L^{-1} d^{-1}), PHA (q_{PHA}, gVSS$_{PHA}$ L^{-1} d^{-1}) and glycogen (q_{GLY}, gVSS$_{Glycogen}$ L^{-1} d^{-1}) and consumption rates of substrate (-q_S, mgCOD L^{-1} d^{-1}) are determined by multiplying the amount of compound produced in the reactor (PHA, glycogen, H$_2$) to the substrate consumed by the dilution rate of the reactor (Outflow rate divided by reactor volume).

3.3.3. ENERGY AND MASS BALANCE ASSESSMENTS

An energy evaluation is performed to check the scalability of the proposed photobiorefinery. The energy balance of the process is assessed to ensure the

sustainability and economic viability of the process (Avellar and Glasser, 1998). Energy balances are carried out simulating a commercial Cambi® CHP plant. Recovery of heat from the flash vapors (saturated steam at 105 °C) to the pre-heating stage of the substrate leads to considerable saving in energy consumption. This way, steam requirements have been estimated with energy and mass balances considering 20% vapor losses in the pre-heating stage according to *Scenario three* in Cano *et al.* (2014). Biogas generation are extrapolated from laboratory scale BMP tests, but steam consumption and equipment's characteristics has been determined theoretically considering typical design values. Briefly:

The biogas is burned in a combined heat and power system (CHP) providing three main streams (Figure 3.5):

Electrical green energy: to be sold, providing net benefits.

Hot exhaust gases (EG): residual stream in which heat can be recovered in a boiler to produce steam for the energy requirements.

Hot water (HW): it can be used to heat the digester, if necessary.
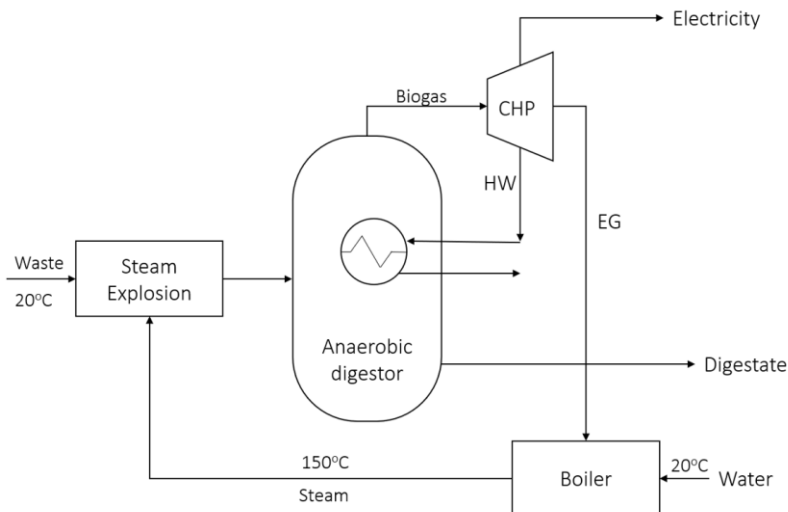

Figure 3.5 Simplified flow diagram with the considerations for the energy balance.

Thermal energy is not necessary for the process that uses directly raw waste since it does not involve a pretreatment process. The total energy of biogas is calculated based on 1 tonne of feedstock (20% TS) and then calculating the amount of biogas produced per kg of TS obtained during the

BMP tests and multiplied by the calorific value of methane. The electrical and thermal balances are estimated by subtracting the thermal needs of the hydrothermal reactor and anaerobic digestor and the electrical needs of the centrigugation, pump and mixing. An overview of all parameters is summarized in Table 3.2. Combining the experimental, theoretical calculations, and published references used in these estimations (Table 3.2), preliminary mass balances were constructed to demonstrate the potential benefits of the proposed process and to estimate the mass yields to the target products (mainly PHA and H_2).

Table 3.2. Mass and energy assessment parameters

Parameter	Unit	Value	Reference
Electrical Efficiency	%	30	(Cano *et al.*, 2014)
Thermal Efficiency	%	55	(Cano *et al.*, 2014)
Energy loses	%	15	(Cano *et al.*, 2014)
Boiler efficiency	%	100	(Cano *et al.*, 2014)
Anaerobic digestion Temperature	°C	37	This study
Steam Explosion Temperature	°C	120-180	This study
Specific heat of water	kJ kg°C^{-1}	4.18	(Safoniuk, 2004)
Heat recovery	%	85	(Lu *et al.*, 2008)
Pumping energy consumption	kJ m^{-3}	1800	(Lu *et al.*, 2008)
Mixing energy consumption	kJ m^{-3}	300	(Lu *et al.*, 2008)
Calorific value of methane	kWh Nm^{-3}	11	(Cano *et al.*, 2014)
Membrane retention	%	100	(Davis *et al.*, 2016)
Centrifugation retention	%	72	(Davis *et al.*, 2016)
Pretreatment rejection	%	10	(Andreasi Bassi *et al.*, 2021)
PHA extraction efficiency	%	90	(Andreasi Bassi *et al.*, 2021)
Prices of electrical energy	€ kWh^{-1}	0.15	(Cano *et al.*, 2014)

3.4. REFERENCES

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CHAPTER 4: RESULTS AND DISCUSSION

SECTION I: PROOF OF CONCEPT

Preamble and rationale

This section of the dissertation focused on the treatment of OFMSW and LW is studied through the concatenation of three processes:

1. **Thermal hydrolysis** pretreatment followed by a separation of the liquid and solid fractions
2. **Anaerobic digestion** of the solid fraction
3. **Photo-biological conversion** of the liquid fraction with mixed cultures of PPB.

In this section, we have discussed the rate of hydrolysis of organic matter to the liquid fraction during pretreatment; analyzed the methanogenic potential of the remaining solid fraction, and settle whether a mixed culture of PPB can grow and transform the complex liquid fraction into high value-added products such as proteins, H_2 and PHA. This approach has been tested on a laboratory scale in batch tests. Energy requirements and the yield of the different products was conducted by means of preliminary mass and energy balances in order to validate the proof of concept.

Experimental design

OFMSW (Pre-sorted and selective) was used in section I.1 and LW in section I.2. Prior to thermal hydrolysis pretreatment, the amount of water was adjusted to 20% TS in order to be able to compare results. In section I.1 the pretreatment at 180°C is studied, since it is the optimum temperature determined by Cano and three reaction times: 5, 15 and 30 min. Seeing that the reaction time does not affect the results too much, in section I.2 three temperatures (120°C, 150°C and 180°C) were studied at a reaction time of 5 min. After thermal pretreatment the effluent is centrifuged to separate the solid and liquid fractions and stored at 4°C until use. The solid fraction is used in BMP tests and the liquid fraction in SPA tests. Together with the SPA tests of

the liquid fraction, a control with MOM medium is studied. Table I.1 shows a summary of the experimental design.

Tabla I.1 Summary of the experimental design used in section I.

Variable / Process	Section I.1	Section I.2
Waste	Pre-Sorted and Selective OFMSW	LW
Thermal hydrolysis	Batch reactions at 180 °C Reaction times of: 5, 15 and 30 min	Batch reactions at 120 °C, 150°C and 180°C and a reaction time of 5 min
Anaerobic digestion	BMP batch tests of the untreated waste and the 3 solid fractions	BMP batch tests of the untreated waste, the 3 treated wastes (liquid + solid) and the 3 solid fractions.
Photoheterotrophic process	SPA batch tests of the 3 liquid fractions and control with the same inoculum and optimized media growth.	SPA batch tests of the 3 liquid fraction and control with the same inoculum and optimized media growth.

The results of this section have been published in the following papers:

Allegue, L. D., Puyol, D., and Melero, J. A., (2020). Novel approach for the treatment of the organic fraction of municipal solid waste: Coupling thermal hydrolysis with anaerobic digestion and photo-fermentation. Science of the Total Environment (2020), 714, 136845.

Allegue, L.D., Ventura, M., Melero, J.A. and Puyol, D., (2021). Integrated sustainable process for polyhydroxyalkanoates production from lignocellulosic waste by purple phototrophic bacteria. GCB Bioenergy, 13, 862-875.

Allegue, L. D., Puyol, D., and Melero, J. A, (2020). Food waste valorization by purple phototrophic bacteria and anaerobic digestion after thermal hydrolysis. Biomass and Bioenergy, 142, 105803.

SECTION I.1

4.I.1. OFMSW PROOF OF CONCEPT

In this part of the dissertation, we have analyzed the treatment of the OFMSW with different origins: a fraction pre-sorted by the citizens (denoted as pre-sorted) and the other one sorted by the waste treatment plant (denoted as selective). The temperature selection was based on previous works (Cano *et al.*, 2014), while reaction time was set for 5, 15 and 30 min. Table 3.1 shows the main macroscopic properties of both feedstocks. Surprisingly, there is no significant difference between both raw wastes, at least in macroscopic terms. Nevertheless, we have decided to carry out the study with both wastes to elucidate if some other compounds could affect the performance of the coupled process and if there is any benefit of the pre-sorting in origin by the citizens.

4.I.2. THERMAL HYDROLYSIS

Table I.2 summarizes the macroscopic characteristics of the liquid and solid phases after thermal treatment at different times and at 180 °C for pre-sorted and selective OFMSW. VS destruction efficiencies ranged from 27-30% and 37-38% for pre-sorted and selective OFMSW, respectively. On the other hand, COD, N, and P solubilization averaged 40% and 38%, 11% and 12%, and 27% and 25%, respectively, for pre-sorted and selective OFMSW, respectively. These values led to a very high COD/N/P mass ratios (100/0.62/0.72 on average), within the liquid phase, which theoretically benefits the accumulation of carbon in the form of PHA by PPB (Fradinho *et al.*, 2019).

No statistically relevant differences were found in regards to the three different hydrolysis reaction times. High COD and low nutrient release is achieved independent of the reaction time. Nevertheless, a more comprehensive characterization of the hydrolysate, in terms of proteins, carbohydrates, lipids, volatile fatty acids, sugars, etc. composition is necessary for a better understanding of the reaction mechanism. Although this subject is encouraged, it is out of the scope of this proof-of-concept study.

Table I.2. Macroscopic characteristics of raw OFMSW samples and solid and liquid fractions after the hydrothermal pretreatment under different temperatures.

		Pre-sorted				Selective		
	Raw	5'	15'	30'	Raw	5'	15'	30'
TS (g kg^{-1})	367 ± 51	245 ± 26	243 ± 82	263 ± 62	343 ± 42	201 ± 8	240 ± 24	207 ± 33
VS (g kg^{-1})	298 ± 46	214 ± 23	211 ± 83	218 ± 33	297 ± 19	187 ± 28	182 ± 26	181 ± 22
COD (g kgTS^{-1})	112 ± 12	89.2±8.5	95.3 ± 3.5	94.1 ± 3.3	135 ± 7	85.7 ± 4.5	87.9 ± 2.5	89.1 ± 4.4
TP (gP kgTS^{-1})	1.2 ± 0.1	0.9 ± 0.5	1.0 ± 0.7	1.0 ± 0.6	1.1 ± 0.6	0.9 ± 0.5	1.0 ± 0.4	0.7 ± 0.4
TKN (gN kgTS^{-1})	3.7 ± 1.7	3.2 ± 0.1	3.4 ± 0.2	3.2 ± 0.1	3.5 ± 0.8	3.2 ± 0.1	3.1 ± 0.1	3.0 ± 0.2
Liquid fraction								
TSS (mg L^{-1})		0.95 ± 0.21	1.10 ± 0.18	1.05 ± 0.11		1.21 ± 0.08	1.14 ± 0.11	1.16 ± 0.13
VSS (mg L^{-1})		0.94 ± 0.1	1.07 ± 0.12	1.02 ± 0.17		1.18 ± 0.05	1.11 ± 0.09	1.13 ± 0.08
TKN (mgN L^{-1})		446 ± 5	351 ± 2	423 ± 5		495 ± 4	483 ± 5	377 ± 3
TP (mgP L^{-1})		675 ± 40	684 ± 34	694 ± 41		545 ± 35	512 ± 21	578 ± 41
TCOD (g L^{-1})		54.2 ± 1.5	54.5 ± 3.2	61.4 ± 3.5		51.7 ± 1.5	51.6 ± 1.5	51.8 ± 1.3
SCOD (g L^{-1})		53.9 ± 0.4	53.3 ± 0.8	61.9 ± 0.8		50.2 ± 0.3	50.7 ± 0.4	51.9 ± 1.4
NH$_4^+$ (mgN L^{-1})		269 ± 8	253 ± 9	227 ± 10		227 ± 6	281 ± 18	294 ± 36
PO$_4^+$ (mgP L^{-1})		338 ± 178	514 ± 170	567 ± 214		365 ± 98	307 ± 73	373 ± 66

4.I.3. ANAEROBIC DIGESTION

The potential for product recovery from the solid fraction upon thermal hydrolysis is performed by analyzing their BMP and the metals and nutrients composition of the digestate. Figure I.1 depicts the time course of the methane potential of different remaining solids after pretreatment and the fresh waste untreated (raw). The methanogenic potential is referenced to the initial concentration of VS before the hydrolysis in the raw waste (VS$_o$), thus including into the story line the effect of the removal of the liquid fraction. A maximum of 253 ± 11 and 246 ± 20 LCH$_4$ gVS$_o^{-1}$ were obtained for pre-sorted and selective

biowastes respectively after pretreatment. The results are compared with a control test where anaerobic digestion was performed to the untreated OFMSW and where all the mass of the residue sum up for the methane production potential. As expected, methane production values of the treated samples are lower than those from the fresh solids that yielded 320 ± 15 and 342 ± 22 LCH$_4$ gVS^{-1} respectively. As seen in the previous section, the removal of the liquid fraction upon hydrolysis led to 30-45% of solids destruction, probably the most biodegradable part, and consequently affecting the reduction of the BMP. Nevertheless, these results are within the range of similar studies reported in the literature: ranging from 180 to 570 LCH$_4$ gVS^{-1} (Bala *et al.*, 2019; Cabbai *et al.*, 2013; Campuzano and González-Martínez, 2016; Cano *et al.*, 2014). Hence, the effect of liquid removal upon the hydrolysis did not imply a significant reduction of the methane potential of the remaining solids.
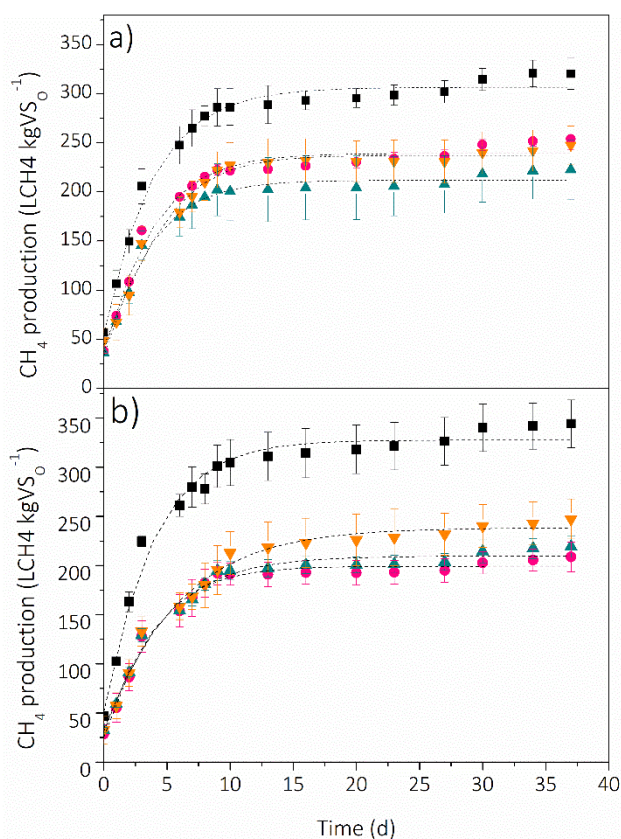

Figure I.1 BMP tests during the anaerobic digestion of the different solid fractions. Pre-sorted OFMSW (a) and Selective OFMSW (b). Initial OFMSW without thermal pretreatment (■), 5' (●), 15' (▲) and 30' (▼) min of reaction time.

Figure I.2 reports the hydrolysis constant (K_h) and the biodegradability extent (B_o), with 95% confidence regions for the conditions of the different tests. Although overall methane production is lower after the pretreatment (Figure I.1), kinetic parameters are quite similar to that obtained from the fresh OFMSW.
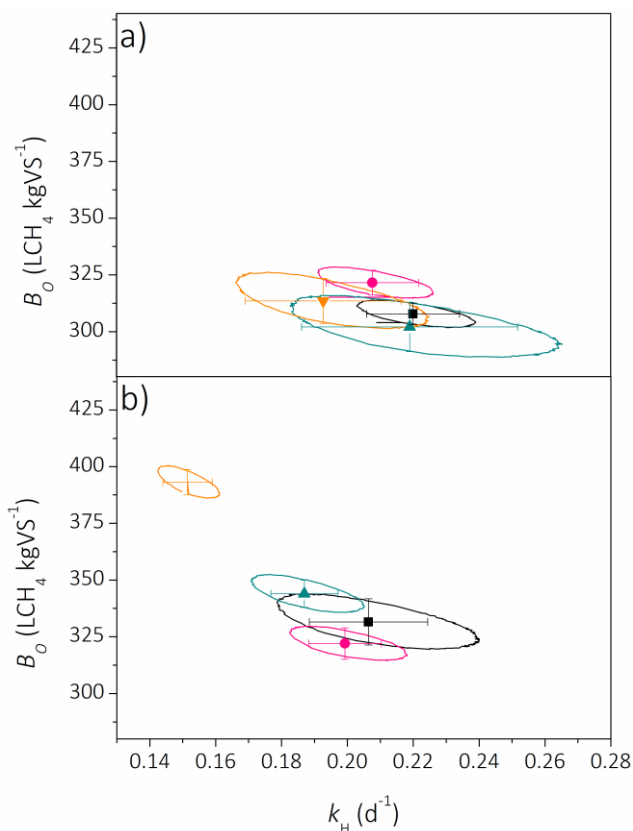

Figure I.2: Results from BMP tests (added feedstock basis). 95% confidence regions for the first-order kinetic parameters during the BMP tests of the solid fraction pre-sorted OFMSW (a) and Selective OFMSW (b). Initial OFMSW without thermal pretreatment (■), 5' (●), 15' (▲) and 30' (▼) min of reaction time.

This demonstrates that thermal hydrolysis pretreatment is increasing the anaerobic biodegradability of the less biodegradable fraction, thus it increases the total profitability of the proposed treatment. Results show no statistically relevant differences between thermal hydrolysis times for OFMSW except for 30 min reactions on the selective OFMSW, which was slightly improved probably due to a small difference in the homogeneity of the raw samples. It must be stated that the main goal of thermal hydrolysis is to obtain an organic liquid feedstock with a high COD/N/P ratio. The benefit obtained with the

treatment of the liquid fraction is key to check on the viability of the proposed approach, as will be furtherly discussed.

In addition to the energy potential of biogas, anaerobic digestion also produces a digestate that must be studied for its possible applications. The use of digestate from stabilized OFMSW has been shown to have benefits to soil, crops, and the environment (Prabpai *et al.*, 2009) due to the low organic content of the residual solid after digestion. In our results, a minimum of 40% VS and 70% COD removal was achieved after anaerobic digestion. However, the digestate must comply with some requirements before it is used as a marketable product. First, the digestate must be free of pathogens before land application. The pathogens reduction has not been checked in this work. However, it has been demonstrated that a 180 ^{0}C thermal hydrolysis is very effective for the pathogen's reduction (Ruiz-Espinoza *et al.*, 2012). Second, urban waste may contain high levels of heavy metals, which is of concern for a fertilizer due to potential soil fertility deterioration, ground-water quality damage, and food chain contamination (McBride, 1995). Metal content in the solid digestate was measured and the results were compared to the Spanish law (Real Decreto 506/2013) that regulates organic fertilizers (see detail in Table I.3).

Table I.3 Metals and nutrient composition of the digestate samples upon hydrolysis and centrifugation compared to the Spanish legislative limits for organic fertilizers.

	Measured data (mg kg $^{-1}$)		Limit values RD 506/2013 (mg kg $^{-1}$)		
	Pre-sorted	Selective	Class A	Class B	Class C
Cd	0.9	1.6	0,7	2	3
Cu	134	277	70	300	400
Ni	20	21	25	90	100
Pb	58	39	45	150	200
Zn	104	466	200	500	1000
Hg	0.5	1.1	0.4	1.5	2.5
Total N (%, d.b.)	1.9 ± 0.5	2.4 ± 0.4			
P_2O_5 (%, d.b.)	0.28 ± 0.02	0.27 ± 0.03			

Considering the metals composition of the final digestate, it may be registered as a Class B fertilizer, which is not subject to limitations on its use. It seems that the pre-sorted OFMSW has better properties than the selective OFMSW regarding metal composition and its values are close to limit values to be considered as Class A fertilizer. Thereby, an improvement of the pre-sorting efficiency (through e.g. better awareness campaigns) can decrease the metal

contamination of the residue and may benefit the quality of the produced fertilizer. Finally, selective OFMSW had a higher nitrogen content on dry mass, just above the 2% necessary to be considered organic fertilizer type N, as specified in the Spanish law aforementioned (Real Decreto 506/2013). Thereby, depending on the initial composition of the OFMSW the digestate can be potentially considered as an organic fertilizer or as an organic amendment, closing the carbon and nutrient cycle.

4.I.4. SPECIFIC PHOHETEROTROPHIC ACTIVITY TESTS (SPA)

Growth process

Standard SPA tests were performed on the liquid fraction upon thermal hydrolysis. Figure I.3 shows the results about COD consumption and biomass growth in terms of mgVSS^{-1}. The control test that used the same inoculum but grown in the MOM served as a reference for the maximum theoretical specific growth rate. Active biomass assimilated between 59-82% of the SCOD in the experimental tests. The experiments lasted 300 h for both sources of OFMSW with more than 80% of the consumed SCOD assimilated in the first 200 h and a biomass growth up to 400 mgVSS L^{-1}.
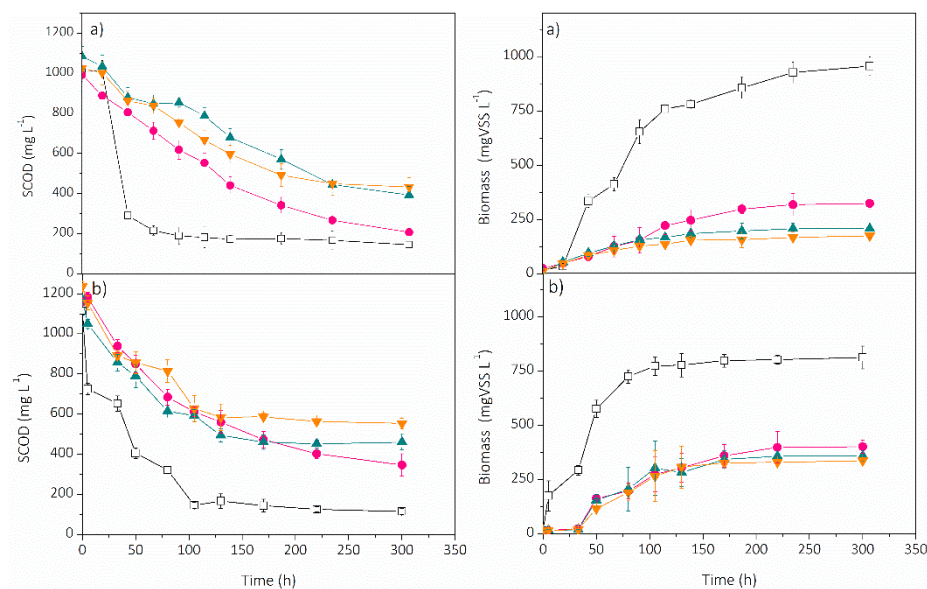

Figure I.3 Comparison of SCOD consumption and biomass growth in the SPA tests using the liquid fraction. Results tests for pre-sorted OFMSW (a) and selective OFMSW (b) at 5' (●), 15' (▲) and 30' (▼) min are compared with a control experiment (□) with the same inoculum.

Near to 100% of main nutrients (N and P) were consumed, likely due to their low concentration compared to the organic carbon concentration (see Figure I.4). The low nutrient content is therefore limiting biomass development, especially ammonium. High P removal even upon ammonium depletion, suggests the appearance of accumulative processes (e.g. polyphosphate accumulation), which improves the potential of nutrients recovery and increases the quality of the phototrophic biomass as a product. The pH at the end of all the assays was on average 8.5. As the initial pH was set at 6.5 the pH increase was likely due to consumption of volatile fatty acids by PPB. The liquid fraction after 5 min pretreatment has proven to be the most biodegradable in all cases, whereas there were no relevant statistical differences between 15 and 30 min of pretreatment. The release of poorly biodegradable organics due to long hydrolysis times may be the main reason for the lower biodegradability of the soluble organics. This fact has been previously related to Maillard reactions, which form recalcitrant melanoidins at temperatures higher than 160 °C (Dwyer *et al.*, 2008).
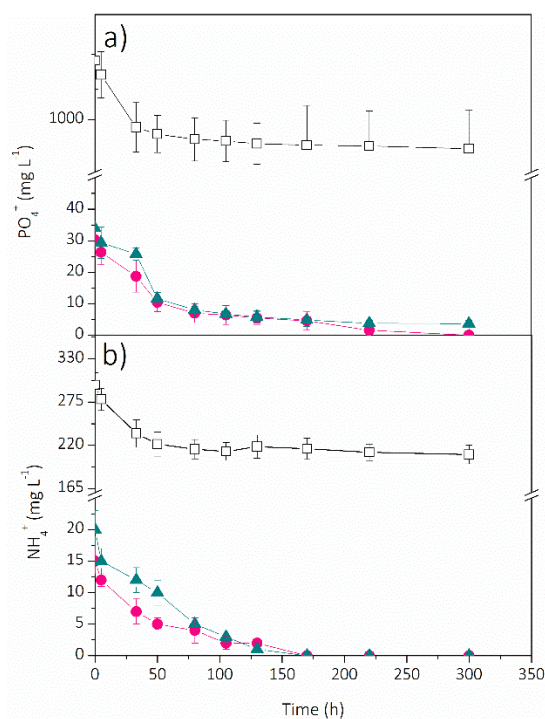

Figure I.4: Comparison of PO_4^{3-} (a) and NH_4^+ (b) consumption in the SPA tests using the liquid fraction (Average of the 3 different thermal hydrolysis reactions). Results tests for pre-sorted OFMSW (●) and selective OFMSW (▲) are compared with a control experiment (□) with the same inoculum.

In the SPA tests, initial and final biomass samples showed prominent VIS/NIR absorption peaks at 590, 805, and 865 nm in all cases, corresponding to the typical absorption spectra of bacteriochlorophyll a, thus confirming the dominance of the PPB within the MMC culture under IR light, which concurs with previous studies (Hülsen *et al.*, 2014).

The quantification of the photoheterotrophic metabolism was assessed by calculating the specific phototrophic activity (k_M) and the biomass yield ($Y_{x/s}$), respectively (Figure I.5). The k_M of control tests yielded 0.035 ± 0.005 g SCOD gVSS^{-1} h^{-1}, and the tests carried out with the two types of OFMSW yielded on average 0.022 ± 0.003 g SCOD gVSS^{-1} h^{-1}. These results are in line with previous studies (Puyol *et al.*, 2017) that reported a k_M of 0.036 g SCOD g VSS^{-1} h^{-1} on acetate for a PPB mixed culture.
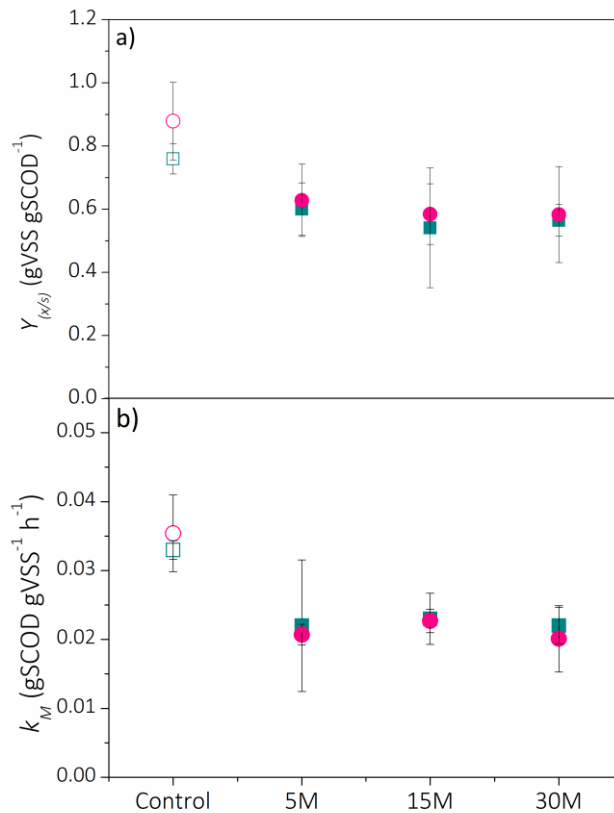

Figure I.5: Comparison of biomass yield ($Y_{X/S}$) (a) and specific phototrophic activity (k_M) (b) in SPA tests using the liquid fraction of every waste upon the thermal hydrolysis at different reactions times. Results tests for Pre-Sorted OFMSW (■) and Selective OFMSW (●) are compared with a control experiment (open symbols) with the same inoculum.

On the other hand, the $Y_{x/s}$ was 0.87 ± 0.07 and 0.75 ± 0.08 gVSS gSCOD^{-1} for the control tests while averages for OFMSW are 0.60 and 0.57 gVSS gSCOD^{-1} for selective and pre-treated OFMSW, respectively. Although $Y_{x/s}$ is lower compared to the control, the biomass yield obtained in these tests were higher than the values reported in the literature (up to 0.56 gVSS gCOD^{-1}) for the treatment of domestic wastewater (Dalaei *et al.*, 2019) and poultry processing wastewater (Hülsen *et al.*, 2018) with PPB. Biomass yield values lower than the controls suggest the presence of non-phototrophic organic transformations (e.g. fermentation or anaerobic oxidation processes), as in essence photoheterotrophy implies almost a complete conversion of organics into biomass (Hülsen *et al*, 2016).

Moreover, the hydrolysis reaction times caused non-significant differences over the two metabolic parameters, which means that the lower biodegradability due to the extended hydrolysis time did not cause toxicity over the PPB metabolism. It must be noteworthy that the inoculum was not adapted to the feedstock. Thus, the ability of non-adapted PPB to grow on these organic sources demonstrates the feasibility of the proposed concept.

Tests Products

During SPA tests, PHA and biohydrogen productions were measured, and SCP production was estimated based on biomass production. Figure I.6 (a) shows a maximum of 5.1 ± 0.3% (wt.) of PHA production yield (Y_{PHA}) for the pre-sorted OFMSW upon 5 min of thermal hydrolysis and a minimum of 2.0 ± 0.1% (wt.) for the selective one upon 30 min thermal hydrolysis. PHA production in control assays was slightly higher, averaging 5.8 ± 1.9% (wt.). Data shows that PHA production is not depending on the thermal hydrolysis times, as there are no clear trends of statistical relevance. Low PHA content (even in controls) is due to the sampling at the end of each essay, instead of at the time of the peak of maximum PHA production. In any case, the PHA yields obtained are considerable, stating that the inoculum is a PPB mixed microbial culture, and are within the lower range found in the literature (3 to 30%), even in works where an adapted phototrophic consortium was used (Fradinho *et al.*, 2016, 2019). Therefore, **these results are promising and encourage future research to maximize the production of PHA in a continuous process using the OFMSW as an efficient and low-cost feedstock.**
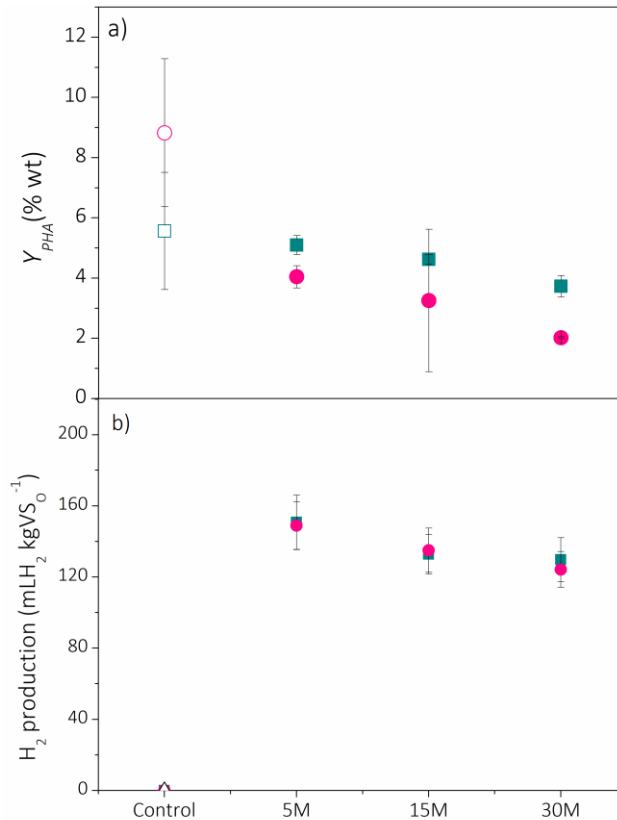
Figure I.6: Comparison of PHA production yield (Y_{PHA}) (a) and H_2 production (b) in SPA tests using the liquid fraction of every. Results tests for pre-sorted OFMSW (■) and selective OFMSW (●) are compared with a control experiment (open symbols) with the same inoculum.

Figure I.6 (b) shows the H_2 production along with the experiment. The maximum production was achieved upon 5 min of thermal hydrolysis (150 ± 15 mLH_2 kgVS$_0^{-1}$), whereas the minimum production was achieved upon 30 min of thermal hydrolysis (124 ± 10 mLH_2 kgVS$_0^{-1}$). These data mean an average production of 0.071 LH_2 L^{-1} on the hydrolysate. The thermal hydrolysis times caused non-significant differences. The controls had no hydrogen evolution due to the high ammonium content, which inhibits the hydrogen production in the nitrogenase complex (Sasikala *et al.*, 1993). H_2 production is lower than recently published results, where values ranging from 0.4 to 8.6 L H_2 L^{-1} of waste have been reported (Ghosh, *et al.*, 2017). Low H_2 production responds to the presence of ammonia at the beginning of the assay and the rise of pH above 8, which is also a key factor along with temperature in H_2 production with phototrophic bacteria (Bolatkhan *et al.*, 2019). In any case, these results,

although modest, may open the opportunity for future research in the optimization of H_2 production using this feedstock. A combination between dark fermentation and photo-fermentation may be a promising option as it has been described previously (Argun and Kargi, 2011). In addition, H_2 is not generated directly as a result of a metabolic pathway but occurs as a mechanism for controlling the reducing power through the nitrogenase enzyme complex (Kotay and Das, 2008).

Finally, SCP estimation content was performed following the literature (Eding *et al.*, 2006). PPB uses IR light as a catabolic driver and enables a non-destructive, assimilative uptake of organics, nitrogen, and phosphorous (Hülsen, *et al.*, 2016). Recent studies show that in a medium with high salinity or high concentrations of ammonium, PPB develops specific proteins as a method of defense against chemical stress (Delamare-Deboutteville *et al.*, 2019). On one hand, content in control assays was high, up to 777 ± 31 mgSCP gVSS^{-1}, likely due to excess ammonium in the media. These results are in line with a recent study in which a maximum of 720 mgSCP gVSS^{-1} was obtained using pork flush as a substrate, which also has a high nitrogen content (Hülsen *et al.*, 2018). The PPB mixed cultures from the OFMSW assays show a lower protein content yield of up to 482 ± 21 and 421 ± 19 mgSCP gVSS^{-1} for pre-sorted and selective OFMSW, respectively. These results are in agreement with the low biomass growth and ammonium content of these tests. Nitrogen is a key factor in protein synthesis, and its deficiency in the media causes a low protein content. However, a recent study showed protein content in PPB averaging 45%, but a production rate up to 1.7 g dry weight $L^{-1} d^{-1}$ of SCP was possible by using a wise combination of synthetic VFAs (Alloul *et al.*, 2019). **This makes PPB an economically viable alternative source of microbial protein in contrast with the current conventional agricultural-based supply route for nutritive animal proteins which has a high environmental and water impact.** This alternative route is a more environmentally friendly way of protein production that can be optimized using the OFMSW as a feedstock.

4.I.5. MASS AND ENERGY BALANCES

Energy balance

The energy balance of the thermal hydrolysis pretreatment was assessed extrapolating the BMP results from laboratory tests. Thermal hydrolysis

requires a large amount of thermal energy which is one of the main limitations of the process to be economically feasible (Passos and Ferrer, 2015). A simplified energy balance (see Figure 3.5) has been carried out by simulating a CHP system following the scenarios of energetic integration proposed in Cano *et al.* (2014) and Murphy and McKeogh, (2004). Table I.4 summarizes the energy balances for the different scenarios in terms of electrical and thermal balance (that means energy produced in the CHP system minus energy requirements in the overall process). Test controls, which are the biowastes directly undergoing anaerobic digestion, yielded the highest results in thermal (341 and 367 kWh tonne^{-1} for pre-sorted and selective OFMSW, respectively) and electrical balances (190 and 206 kWh tonne^{-1} for pre-Sorted and selective OFMSW, respectively). The value of electrical and thermal outputs for the assays with pretreatment ranged from 128 to 103 kWh tonne^{-1} and 70 to 23 kWh tonne^{-1}, respectively. These values are lower than that shown by the control sample due to the thermal energy requirements necessary in the hydrolysis step. But all the scenarios independent of the raw material and the hydrolysis time show a positive energetic balance.

Table I.4 Energy integration balance. Results were simulated for CHP system for electricity and thermal energy production. Data referenced to a tonne (t) of FORSU.

Substrate	Total Energy biogas kWh t^{-1}	Electrical Output kWh t^{-1}	Thermal Output kWh t^{-1}	Electrical balance kWh t^{-1}	Thermal energy balance kWh t^{-1}	Electric output Euro t^{-1}
Pre-sorted Control[a]	619	205	341	190	341	29
Pre-sorted 5'	491	163	270	128	70	19
Pre-sorted 15'	431	143	237	108	26	16
Pre-sorted 30'	479	159	263	124	43	19
Selective Control[a]	666	221	367	206	367	31
Selective 5'	417	138	230	103	30	15
Selective 15'	424	141	233	106	23	16
Selective 30'	478	159	263	124	43	19

[a] Direct anaerobic digestion (AD)

[b] Electrical energy produced minus that required for the centrifugation and mixing for AD.

[c] Thermal energy produced minus that required for pretreatment and AD.

These findings are analogous to the results reported in the literature (Cano *et al.*, 2014; Dasgupta and Chandel, 2019). Further optimization of different pretreatment conditions with respect to energy integration (e.g. temperature, pressure, reaction time, or moisture) is mandatory in order to determine the scalability of hydrothermal pretreatment of OFMSW prior to anaerobic digestion and photo-fermentation.

Mass balance

This section shows the preliminary analysis of the global mass balance of the process on initial total solids in the raw OFMSW. Figure I.7 shows the total mass balance of the proposed approach. As a general finding, a maximum of 15% of the initial TS become PHA, H_2, and biomass jointly to microbial protein upon thermal treatment with a 59-61% volume reduction of the waste to be disposed of (5-11% more volume reduction as compared with the traditional treatment of an anaerobic digestion process). Figure I.7 clearly shows the diversity of potential products that can be derived from this new concept. Complete and efficient utilization of resources is important for circular economies as a zero-waste policy is the aim of bioprocesses.
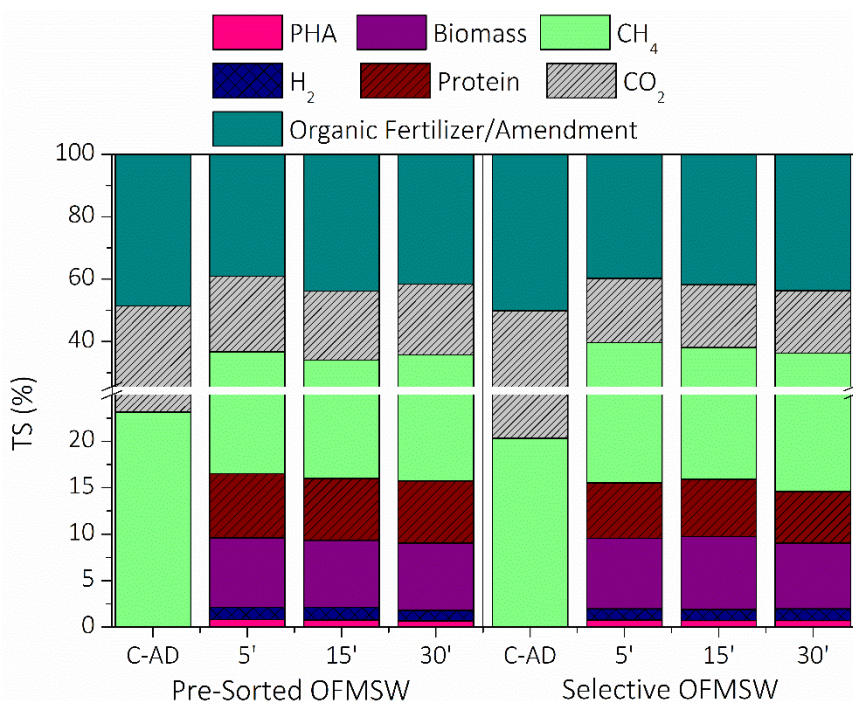

Figure I.6 Mass balance of the overall process at different pretreatment times (5, 15, and 30 min), compared to the traditional direct anaerobic digestion of the wastes (AD).

In order to preliminary analyze the economic viability of this new process, we have compared the selling prices of each individual product. Current benefits are not included and would require a complete social and economic life cycle assessment once the process is conveniently optimized. The biogas produced has been partially used for the energy integration of the process, and only its excess in the production and sale of electricity is considered in this analysis. The incomes expected from electricity are on average 30 € tonne^{-1} for the control tests, and 18 € tonne^{-1} for the assays undergoing the new process. Despite the mass percentage of organic fertilizer being the most important (more than 40% in all cases), its selling price is very low. An average selling price of 4 € tonne^{-1} for certified (RAL) bio-waste fertilizer has been reported in the EU (Meyer-Kohlstock *et al.*, 2015). On the other hand, although PHA constitutes an average of 5.1 g PHA kgTS$_0$$^{-1}$ based on the mass of the residue, its market price is relatively high: 4.5-5.5 € kg^{-1} (Castilho *et al.*, 2009). The final costs of PHA mainly depend on the price of substrates added as a carbon source for microbial growth. Furthermore, the PHA yield on carbon source, PHA productivity, and downstream costs determine their introduction into the global market. The cost of carbon sources was reported to constitute about 50% of the final production cost (Choi and Lee, 1999). Taking this into account, the process proposed uses a virtually cost zero-carbon substrate, adding more interest to the process. Microbial protein constitutes the largest fraction of possible income, due to the large amount of biomass growth produced in photo-fermentation tests. Up to 68 gProtein kgTS$_0$$^{-1}$ was produced. Microbial protein has a market price of 1.1 € kg^{-1} in (Matassa *et al.*, 2016). H$_2$ costs via PPB are considered 10 € GJ^{-1} (Basak and Das, 2007). The purification cost exceeds this figure if we consider that the purity of H$_2$ issued in these batch tests are very low (about 9%). Considering those, H$_2$ is the least economically interesting product of this platform. In any case, the reduction costs derived from the lowering of the waste to be disposed of in landfills, as well as the reduction of the C-footprint, should be put front into an economic life cycle analysis once the concept is verified. Thereby**, the proposed platform may potentially enlarge the economic benefit by targeting PHA and microbial protein production, and future trends should focus on process optimization with these targets in mind.**

4.I.6. Conclusions

This preliminary results confirm the proof of concept for future optimization of an integrated photo-biorefinery process using OFMSW as feedstock. Thermal hydrolysis achieves up to 40% solubilization of the COD depending on the used waste, where the reaction time (5 to 30 min) seems to have low significance over the overall process. The photo-process using PPB mixed cultures has proven to be a resilient technology capable of assimilating organic matter from the OFMSW after pretreatment of thermal hydrolysis, yielding PHA, SCP, and H_2. Different metabolic pathways can be favored to produce these products in specific cases, so the proposed treatment concept is highly versatile depending on factors such as market needs, feedstock composition, and environmental conditions. This new process also has interesting economic prospects based on the expected income from the production of high added-value products. Finally, the anaerobic digestion process closes the carbon cycle, reduces the amount of waste and decreases the C footprint and enables an energetically sustainable process, thus embracing this alternative concept within the circular economy framework

SECTION I.2

4.I.7.   Lignocellulosic waste: proof of concept

In this part of the discussion, we have extended the proof of concept using as feedstock LW (in particular gardening and pruning wastes). It must be noted that this type of LW accounts for 20% of the OFMSW. In this way, in this section, we have performed a parallel work to that described in section I.1. As seen in the previous section, pretreatment reaction time is not an especially relevant parameter, therefore, it has been decided to change the reaction temperatures to 120 °C, 150 °C and 180 °C and keeping reaction time of 5 min.

4.I.8.   Hydrothermal pretreatment

Table I.5 shows the main macroscopic characteristics of the raw waste, and the slurry and liquid fractions upon the pretreatment. Thermal conditions were 120 °C, 150 °C, and 180 °C including the temperature gradient which corresponds to a severity factor of 2.1, 3.1, and 3.9, respectively. Previous studies have shown that the optimal severity for maximizing sugar yield is between 3.0 and 4.5 (Silva-Fernandes *et al.*, 2015). In lignocellulosic biomass, the combination of cellulose, hemicellulose, and lignin makes it highly recalcitrant and hinders the accessibility of hydrolytic enzymes to cellulosic components (Duque *et al.*, 2016). T

he extension of hemicellulose solubilization is directly proportional to the severity factor, while cellulose and lignin are usually retained. Xylan and arabinan-based sugars suffer the highest solubilization on a severity factor of 4 (Carvalheiro *et al.*, 2009). The elemental analysis carried out shows that there are almost no changes in the percentage of carbon, nitrogen, or hydrogen. This means that the pretreatment process did not entail the loss of matter by means of an oxidative process.

Table I.5 Macroscopic characteristics of raw LW samples and solid and liquid fractions after the hydrothermal pretreatment under different temperatures.

Parameters*	Raw	120 °C	150 °C	180 °C
TS (g kg^{-1})	920 ± 8	130 ± 105	130 ± 10	116 ± 9
VS (%TS)	94 ± 1	97.1 ± 0.2	96.9 ± 0.3	97.3 ± 0.4
COD (g kg^{-1} TS)	1120± 40	1060 ± 20	1070 ± 20	1050 ± 20
Carbon (%)	47 ± 1	48 ± 1	48 ± 2	49.6 ± 0.6
Nitrogen (%)	0.8 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.1 ± 0.1
Hydrogen (%)	5.9 ± 0.4	5.8 ± 0.1	5.8 ± 0.2	5.8 ± 0.1
Oxygen (%)[a]	45.9	45.3	45.1	43.5
Liquid fraction				
SCOD (g L^{-1})		19.6 ± 0.9	22 ± 1	45 ± 2
Monosaccharids (%)[b]		2.96	2.74	1.23
Other CA (%)[b]		1.15	3.04	3.41
Oligosaccharides, and proteins (%)[b]		95.90	94.22	95.35
TKN (gN L^{-1})		1.2 ± 0.1	1.2 ± 0.2	1.4 ± 0.1
TOC (g L^{-1})		11.3 ± 1.2	12.8 ± 2.2	26.2 ± 1.8
TIC (g L^{-1})		0.4 ± 0.1	0.5 ± 0.2	1.1 ± 0.4
NH$_4^+$ (mgN L^{-1})		610 ± 10	710 ± 10	930 ± 20
PO$_4^{3-}$ (mgP L^{-1})		100 ± 10	122 ± 8	140 ± 10
pH		5.8	5.2	4.8

[a] % of oxygen was calculated from the difference of C, H, and N since the proportion of S was negligible compared to the rest of the major elements.
[b] Percentage calculated based on SCOD. The %CA was calculated as SCOD – (SCCA + monosaccharides).

To study the changes in greater depth over the solid fraction, XRD and FITR analyses were performed. XRD experiments were mainly performed to determine the crystallinity indexes of the samples before and after pretreatment. The crystallinity indexes may indirectly indicate the amorphous phase signal of the biomass such as hemicellulose, cellulose, and lignin domains (Table I.5). The cellulose in lignocellulosic biomass is composed of crystalline and amorphous structures, which have a great influence on enzymatic hydrolysis (O'Dwyer *et al.*, 2007). This influence also anaerobic digestion, as it uses an analogous enzymatic mechanism, which is derived from the extracellular hydrolysis caused by the release of enzymes by hydrolytic bacteria (Azman *et al.*, 2015).

Table I.5 2θ theoretical values

Cellulose type	Amorphous structure	Crystalline structure
I	15-17 º	22-23 º
II	12-13 º	20-22 º

Figure I.8 shows the CrI XRD spectra comparing raw and pretreated samples. The composition of the biomass influences the CrI since hemicellulose and lignin are amorphous while cellulose is crystalline (Jeoh *et al.*, 2007). Raw lignocellulosic waste had a CrI of 68.6%, which increased up to 73.5, 76.8, and 76.7% upon the 120°C, 150°C, and 180°C, respectively. The increase in CrI was caused by the removal of amorphous substances in the biomass, mostly hemicellulose, which exposed the crystalline cellulose core and increased the glucan content in the pretreated solid fraction. As crystallinity of cellulose is incremented by the hemicellulose removal, an increase in the xylose removal, from amorphous or crystalline hemicellulose is observed (Evans *et al.*, 1995).
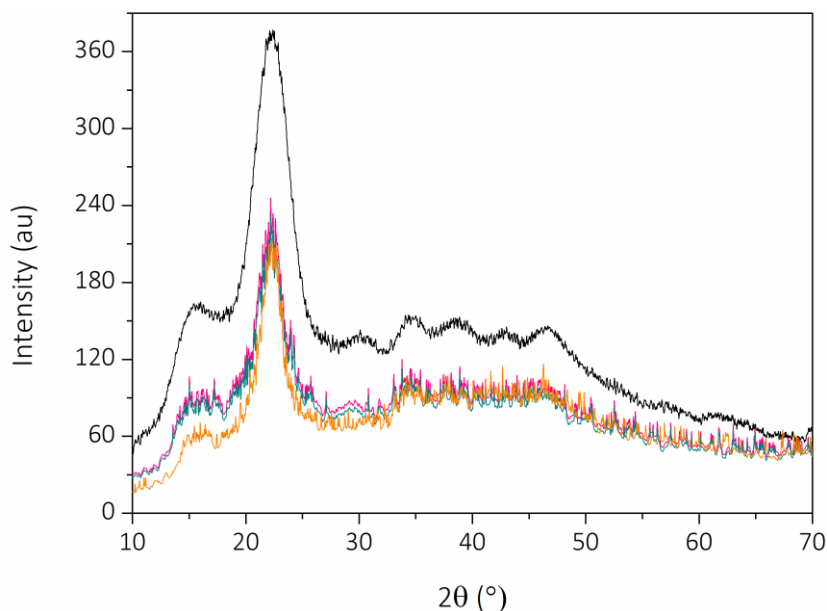

Figure I.8 Comparison of DRX spectra of the raw waste (Black) and the hydrothermal pretreatments solid phases were 120 ºC (Pink), 150 (Cyan) and 180 ºC (Orange).

The FTIR measurements were also used to characterize the solid fraction after pretreatment. The structural changes of hemicelluloses, cellulose, and

lignin after the thermal treatment can be observed in the FTIR spectra. Figure I.9 shows the effect of pretreatment on the change of functional groups of feedstock. The wavenumber at 3252 cm^{-1} shifted to 3759 cm^{-1} with the pretreatment, which indicated the change in the characteristic of crystalline cellulose. The change in the signal was mainly due to less water and hydrogen bonding in the treated biomass. The aromatic ring vibrations of C=C (between 1600-1545 cm^{-1}) changed as temperature increased, which is indicative that the lignin of the biomass was affected. The results observed in the FTIR measurements show clear changes in the crystallinity of cellulose, which agrees with the XRD analyses, partial solubilization of hemicellulose, and slight changes in the structure of lignin. Besides, a clear peak at 1877 cm^{-1} was found in the treated biomass, being more intense as temperature increases. This is related to the acetyl groups of hemicelluloses or the ester linkages of ferulic and p-coumaric acids with lignin (Li and Jin, 2015). Hence, the greater the severity factor the more changes in the crystallinity, solubilization of hemicellulose, and changes in lignin structure.
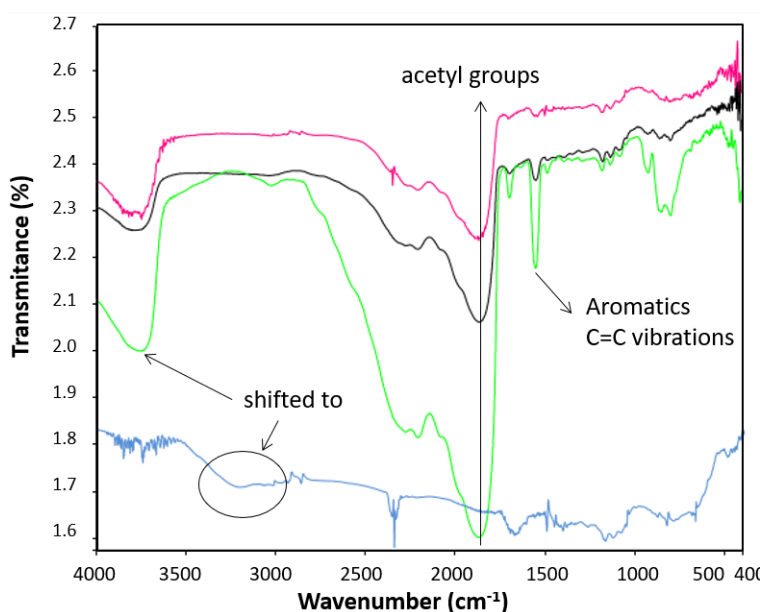

Figure I.9 FTIR spectra of the untreated waste (blue) and the three hydrothermal pretreatments, 120 ºC (Pink), 150 (black) and 180 ºC (green).

The increase of the severity factor also affected the composition and characteristics of the liquid hydrolysate. The solubilization of COD accounts for 9, 14, and 24% for 120 °C, 150 °C, and 180 °C respectively. This means a 69%

increment in COD solubilization for 180 °C compared to 120 °C. A similar trend happens with the destruction of solids, yielding 13, 19, and 29% destruction efficiencies at 120 °C, 150 °C, and 180 °C, respectively. As shown in Table I.4, the pH of the hydrolysate decreased significantly with increasing temperature, due to the presence of SCCA such as acetic and propionic acids that are usually formed during pretreatment of wood biomass (Wang *et al.*, 2018). Values strongly suggest the predominance (about 95% of SCOD) of oligosaccharides and proteins in the liquid phase. Indeed, the liquid fraction obtained after centrifugation had variation in color according to the pretreatment temperature and turned darker at high temperatures likely due to the solubilization of sugars and their subsequent caramelization. Previous works agree with these results and consistently showed that the liquid fraction resulting from the pretreatment of LW like the feedstock used in the current research is mainly composed of oligosaccharides (higher than 95% on a mass basis). Xylose and glucose, followed by galactose, were found as the major components for severity factors similar to the reported in this work, at a temperature of 180 °C. In these conditions, the resulting SCCA concentrations were very low, with values under 1 gCOD L^{-1}, which agrees with our results (Ballesteros *et al.*, 2011; Cara *et al.*, 2006, 2008). Nitrogen solubilization was also dependent on temperature, with up to 1.4 gN L^{-1} at 180 °C, and a 52% increase in inorganic nitrogen content (NH_4^+) compared to the 120 °C. At first glance, 180 °C seems to be the most interesting pretreatment due to the higher solubilization of organic compounds.

4.I.9. BIOCHEMICAL METHANE POTENTIAL TESTS

Figure I.10 shows the results from BMP tests on the raw waste and the solid fractions after pretreatment. An increase in the cumulative methane yield as pretreatment temperature increases is clearly observed. Is important to clarify that after solid-liquid separation by centrifugation the solid fraction was not washed. The effect of thermal pretreatment on the solid fraction of lignocellulosic residues has been extensively studied, for example in (Buitrón *et al.*, 2019), and it is not the scope of this study that attempts to represent results closer to the industrial scale. For that reason, the effect of the pretreatment over the biodegradability of the solid phase alone has been ignored in this work. The raw untreated waste produces an important amount of methane (145 ± 14 LCH_4 gVS_o^{-1}). 180 °C pretreatment resulted in the highest cumulative methane

yield, representing an increase in methane production of 27% compared to the raw sample.
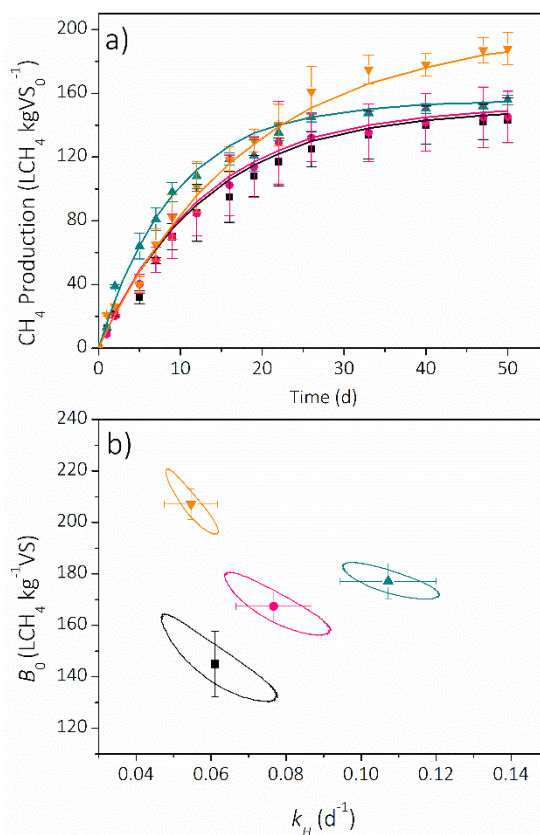

Figure I.10 BMP tests (a) and 95% confidence regions for the first-order kinetic parameters (b) during the anaerobic digestion of the raw waste (■) and the solid fraction after 120 ºC (●), 150 ºC (▲) and 180 ºC (▼) pretreatments.

The increment is noteworthy despite the removal of liquid fraction, which may reduce the methane potential of the sample significantly. Figure I.11 shows that the anaerobic digestion of raw slurry yielded up to 53% higher methane potential than the solid fraction alone, indicating that the removal of the liquid fraction decreased significantly the methane potential. Thereby, the increase of the BMP due to the pretreatment is counteracted with the removal of the liquid fraction. This fact has important implications on the global energy balance of the proposed biorefinery concept, as will be furtherly analyzed.
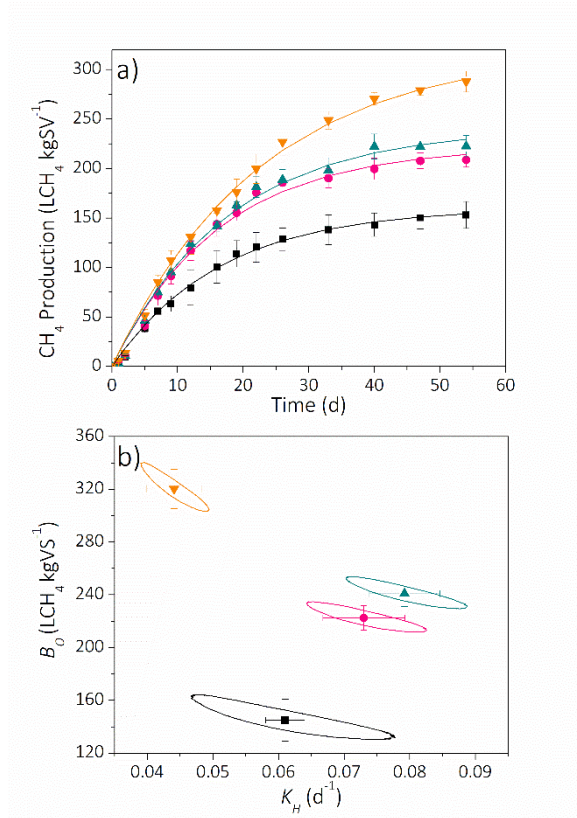
Figure I.11 BMP tests (a) and 95% confidence regions for the first-order kinetic parameters (b) during the anaerobic digestion of the raw waste (■) and the slurry after 120 ºC (●), 150 ºC (▲) and 180 ºC (▼) pretreatments.

The experimental results from BMP tests have been fitted to a simple first-order model following Batstone *et al.* (2003). The 95% confidence surfaces for the hydrolysis constant (k_H) and the biodegradability extent (B_O) were calculated (Figure I.10 and I.11, respectively). Results are presented by using the real feedstock added as a basis (kg VS added), thereby the direct (non-relative) kinetic parameters are representative of the anaerobic digestion process. Estimated values generally predicted accurately the experimental data and confirms the positive relation of the pretreatment temperature with the increase of the biogas production. As an example, the B_O of the experiment using the solid fraction upon 180 °C is 210 ± 10 mLCH$_4$ kgVS^{-1}, which represents an improvement of 31% with respect to the value estimated for the raw waste (146 ± 10 mLCH$_4$ kgVS^{-1}).

The estimated value for the k_H of the untreated sample was 0.060 ± 0.003 d^{-1}, while treated samples reached 0.078 ± 0.002, 0.113 ± 0.004 and 0.057 ± 0.005 d^{-1} for 120 °C, 150 °C and 180 °C, respectively. This means that the pretreatment increased the process rate at 150 °C but maintained at 180 °C despite the increment of the BMP. This trend is even more clear observing the results when using both solid and liquid fraction as feedstock (Figure I.11): in the 180 °C experiment, the B_O increased by 128% (320 ± 10 mLCH$_4$ kgVS^{-1}) but k_H decreased by 33% (0.045 ± 0.002 d^{-1}) with respect to the untreated biowaste. This reduction of the hydrolytic activity for high temperatures would be linked with the formation of inhibitory furan components, which have been reported for temperatures close to 180 °C (severity factor around 4) (Steinbach *et al.*, 2019). Indeed, after removing the liquid fraction, B_O decreases but k_H increases, which clearly points towards the solubilization of a large portion of the organic fraction, but also the presence of potentially inhibitory organics. Hence, the increase of the severity may amplify the formation of these compounds, making the organic matter recalcitrant and less biodegradable. Thereby, temperatures higher than 180 °C are not recommended for this biorefinery platform.

4.I.10. SPECIFIC PHOTOTROPHIC ACTIVITY TESTS

Growth process

Figure I.12 shows the time course of the SCOD consumption and biomass concentration in the SPA tests. A control test under Ormerod media and acetate as organic carbon served as a reference for the maximum theoretical SPA value. Active biomass assimilated 95% of the SCOD in the control test, which was reduced to 55% and 48% in the 120 and 150 °C tests, respectively. This caused an increase of the biomass concentration at the end of the test up to 1046 ± 30 mgVSS L^{-1} in the control test and 649 ± 20 and 640 ± 30 mgVSS L^{-1} in the 120 and 150 °C. A considerable reduction occurred for the 180 °C, where just 37% of SCOD was consumed and biomass concentration reached 490 ± 30 mgVSS L^{-1}. In parallel, Figure I.13 shows that close to 100% of the main nutrients (N and P) were consumed, likely due to their low concentration compared to the organic carbon concentration.
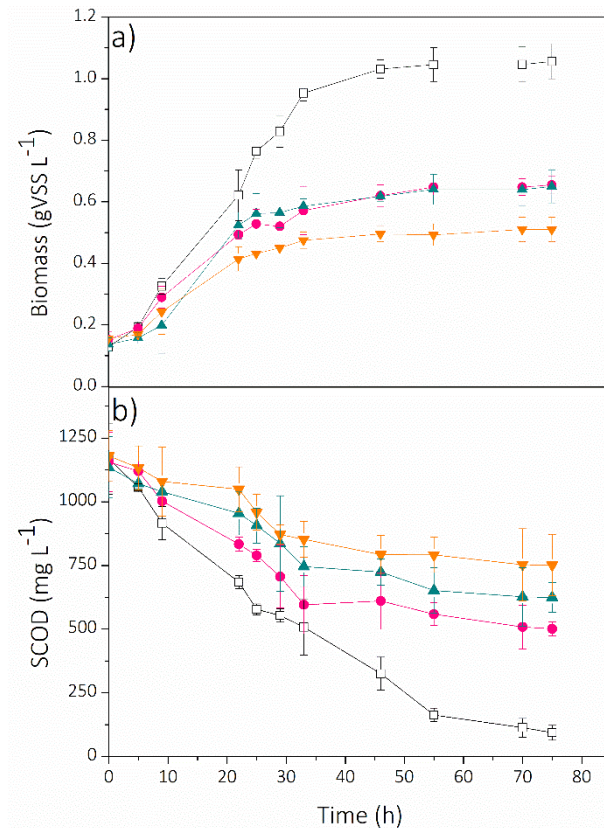
Figure I.12 Comparison of biomass growth (a) and SCOD consumption (b) in the SPA tests using the liquid fraction. Results tests for hydrolyzed at 120 ˚C (●), 150 ˚C (▲), and 180 ˚C (▼) are compared with a control experiment (□) with the same inoculum.

Therefore, the limitation of nutrients that leads to the accumulation of PHA can be due to both N and P. Likewise, some H_2 was produced on the experimental tests, which accounted for 8, 10, and 17 mgCOD L^{-1} at the end of the 120, 150, and 180 ⁰C tests, respectively. H_2 production was coincident with ammonium depletion, and the control experiment did not produce H_2 due to an excess of nutrients. The highest COD/N ratio found at the 180 °C test was coincident with the lowest SCOD consumption and the highest biohydrogen production. This indicates that the nutrient limitation was the main cause of the lower COD consumption efficiency during the phototrophic tests, which was furtherly analyzed through model-based analysis.

In general, the waste composition fits very well with the aim of PHA production by PPB. The main driver for PHA production is the scarcity of nutrients, especially N and P, which limits bacterial growth, pushing PPB to accumulate carbon excess in form of PHA (Monroy and Buitrón, 2020). As

shown in Table I.4, the raw lignocellulosic waste contains a low N proportion, resulting in a COD/N ratio of 100/0.71. Upon pretreatment, the liquid fraction resulted in COD/N/P ratios (considering SCOD, N as NH_4^+ and P as PO_4^{3-}) of 100/3.1/0.5, 100/3.2/0.5, and 100/2.1/0.31 for temperatures of 120, 150, and 180 ⁰C, respectively. The increase of the N proportion in the liquid phase with respect to the raw waste indicated that proteins have been solubilized preferentially during the pretreatment, but while the temperature is rising, more carbohydrates are being solubilized, resulting in lower COD/N ratios. As it has been been shown, this had strong effects on photoheterotrophic growth. The optimum physiological COD/N/P ratio for mixed cultures of PPB is 100/7.2/1.8 (Puyol *et al.*, 2017), though average values in literature are around 100/5/1 (Capson-tojo *et al.*, 2020). Increasing COD to nutrient ratios entails a preferential usage of COD for PHA accumulation, as the medium lacks key nutrients to allow for bacterial growth.

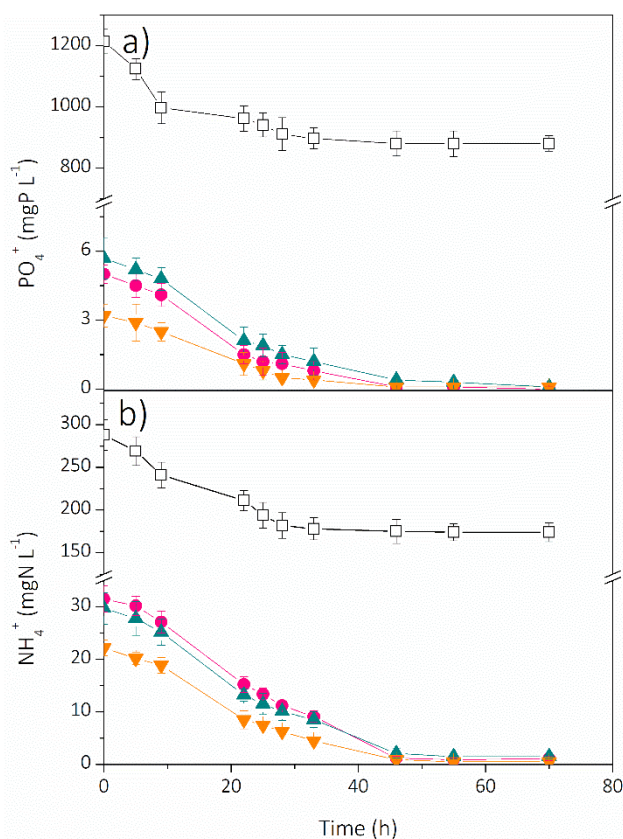**Figure I.13** Comparison of PO_4^+ (a) and NH_4^+ (b) consumption in the SPA tests using the liquid fraction. Results tests for hydrolyzed at 120 ˚C (●), 150 ˚C (▲), and 180 ˚C (▼) are compared with a control experiment (□) with the same inoculum.

Figure I.14 depicts the kinetic parameters of the tests ($Y_{X/S}$ and k_M). The temperature caused a negligible effect on $Y_{X/S}$ while the k_M of the control was significantly higher than found for the treated samples, but the values for the experimental tests were not significantly different from each other. This is likely due to the higher biodegradability of the synthetic media in comparison with the complex hydrolysate. In any case, k_M values for the experimental control tests averaged 0.78 gSCOD gVSS^{-1} d^{-1}, which are in the same range as the values reported in the literature for simple organics (Puyol *et al.*, 2017).
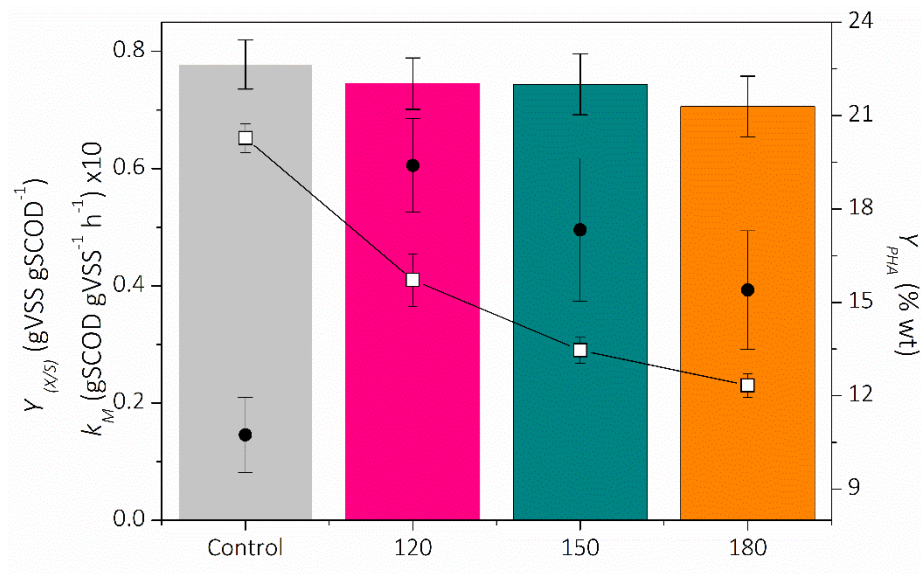

Figure I.14 Biomass yield ($Y_{X/S}$) (columns), specific phototrophic activity (k_M x 10) (□) and PHA production yield (Y_{PHA}) (●) in SPA tests using the liquid fraction at different temperatures. Results tests are compared with a control experiment using the same inoculum.

The differences between the $Y_{X/S}$ calculated for the experiments at the three temperatures were also not statistically relevant, which indicates the solubilization of organic compounds does not affect the $Y_{X/S}$. It can be concluded that the hydrolysate is highly biodegradable by the mixed culture of PPB. As the cultures were not previously acclimated to these effluents, this data strongly supports the viability of the proposed photobiorefinery. It must be noted that the $Y_{X/S}$ values obtained in this work are generally higher than others reported in the literature (Capson-tojo *et al.*, 2020). This is attributed to the partial release of COD in form of hydrogen (as commented previously) and a lower COD/VSS ratio of the biomass produced in this work. The accumulation of PHA had an important role here, as PHA ($[C_4H_6O_2]n$) is more oxidized than

PPB biomass ($CH_{1.8}O_{0.38}N_{0.18}$, (Puyol *et al.*, 2017)) (1.62 vs 1.75 gCOD gVSS^{-1}, respectively). Therefore, a detailed analysis of the PHA accumulation has been conducted.

Tests Products

Figure I.14 shows the PHA production yield (Y_{PHA}) that was measured mid-trial, 30 h after the experiment began when the SCOD consumption in the experiments was around 80%, and at the end of the experiments. The only type of PHA detected was poly-3-hydroxybutyrate (PHB). Although the presence of propionic acid has been related to the production of the copolymer (PHV), its concentration in the experiment after the substrate dilution to 1 gCOD L^{-1} was negligible. Data shows that PHA production is slightly dependent on the thermal hydrolysis temperatures. In the measure taken at 30 h after the experiment began, the Y_{PHA} values averaged 19, 17, and 15% for the 120, 150, and 180 °C, respectively. On the measurement taken at the end of the experiment, on the stationary phase, a slightly reduced Y_{PHA} was noticed: 16, 16, and 14%, which may be related to the consumption of PHA for maintaining the redox balance, as has been recently suggested (Bayon-vicente *et al.*, 2020). Lower Y_{PHA} on the control tests were obtained (10%), which responds to the excess of nutrients in the synthetic medium, as PHA production is enhanced when growth is limited (Brandl *et al.*, 1991). The PHA yields are significantly higher than those found in the literature (5-6.3%) when using PPB MMCs with real waste substrates (Monroy and Buitrón, 2020). This is remarkable taking into account that the inoculum is not adapted to this complex media, which contains a very low amount of VFAs. But it is still far from the 30-40% achieved when PPB MMC have been used with synthetic substrates such as acetate (Fradinho *et al.*, 2019), and up to 70% using acetate and pure cultures (Brandl *et al.*, 1991). **Nevertheless, these results are, to the best of our knowledge, the first instance to report the production of PHA in waste-lignocellulosic hydrolysates using PPB.**

Most of the studies published on MMC agree that acetate and other SCCA are the predominant carbon source for PHA production (Lee *et al.*, 2014; Reis *et al.*, 2003). But the presence of these volatile fatty acids in our liquid fractions were low (1.66% of the SCOD or up to 570 mg L^{-1} of acetic acid and 43 mg L^{-1} of propionic acid for 180 °C). Most of the organic carbon must be derived from the solubilization of the lignocellulosic material (hemicellulose) into oligosaccharides majorly containing xylose, glucose, and arabinose, as

previously discussed. Oligosaccharides can be catabolized by three main pathways to PHB: the Embden-Meyerhof-Parnas (EMP), the Entner-Doudoroff (ED), and the Pentose-Phosphate Shunt (PPS) (Jeffries, 1983). Previous studies have verified symbiotic interaction between species of xylan fermentative bacteria (which contains xylanase) and phototrophic bacteria (Hongyuan et al., 2016), where two main mechanisms may occur. First, fermentative bacteria can produce the xylan hydrolysis, followed by fermentation of simple sugars to VFA (Dziga and Jagiełło-Flasinska, 2015), and the consequent accumulation of PHA by PPB. A second mechanism may be simpler, as PPB can degrade glucose and arabinose (through the EMP pathway) and have also been shown to cause direct degradation of xylose, in fact, the genome of some species indicates the presence of xylose ABC transporter, which suggests that these bacteria actively degrade it via PPS pathway (Pattanamanee et al., 2012). Therefore, xylan hydrolysis by fermentative bacteria can occur, followed by the PPS of the resulting xylose by PPB bacteria, which internally derive the products of xylose degradation to the EMP, and hence, PHA accumulates from acetyl-CoA. The excess of electrons from this process can be assimilated by PPB through the Calvin-Benson-Batham cycle, where this pathway works as an electron sink for attaining redox homeostasis in PPB. This idea agrees with the type of PHA found in all the experiments, which was PHB as reported previously. Future studies on the mechanism of xylan degradation in the photo-heterotrophic process by PPB and PHA accumulation are encouraged but are beyond the scope of this study.

4.I.11. Energy balance

A theoretical energy balance of a full-scale CHP plant was estimated using the experimental data from the batch pretreatment and BMP tests and other parameters from the literature. Thermal pretreatments require a large amount of thermal energy to be carried out and hence it is one of the bottlenecks of the process to be economically feasible (Avellar and Glasser, 1998). As shown in Table I.6, positive thermal and electrical balances were achieved on all temperatures, confirming the energetic viability of the proposed process. Besides, the electrical balance of the process has been calculated, and the remaining electricity can be sold to the electrical market. One of the determining parameters of a THP process is the boiler efficiency of wet biomass that strongly depends on moisture content. We used 20% TS, but it should be

thoroughly studied when scaling up the process and can be adapted for better integration of the process.

Table I.6 Energy integration balance. Results were simulated for a CHP system for electricity and thermal energy production. Data referenced to a tonne (t) of LW.

Substrate	Total biogas energy kWh t^{-1}	Thermal Output kWh t^{-1}	Electrical Output kWh t^{-1}	Electrical balance kWh t^{-1}	Thermal energy balance kWh t^{-1}	Electric output Euro t^{-1}
Raw[a]	1343	739	443	428	739	64
120°C	1343	739	443	408	92	61
150°C	1398	769	461	426	26	64
180°C	1693	931	559	524	49	79

[a] Direct anaerobic digestion (AD)

[b] Electrical energy produced minus that required for the centrifugation and mixing for AD.

[c] Thermal energy produced minus that required for pretreatment and AD.

4.I.12. PROSPECTS AND INDUSTRIAL IMPLICATIONS

PHAs have gained much attention both in research and industry, but their production cost is still their greatest disadvantage. But the approach of the European Commission towards a circular economy strategy and the recommended use of biodegradable plastics through the EU Commission directive 2018/0172 and even more the recent adoption of the European Green Deal, opens the door to new investments and research in this field, especially in Europe.

To improve the PHA production process, industrial producers are currently working towards decreasing the cost price of these biopolymers by increasing the volumetric production capacity of the fermentation systems and improving the process technology. We believe that the combination of lignocellulosic wastes hydrolysates and PPB can help to achieve this goal. Lignocellulosic biomass has been projected as an abundant and promising alternative to replace crude oil, while PPB have several advantages over aerobic fermentation microorganisms, which are currently been used in commercial PHA. The use of PPB eliminates the need for aeration, in addition to its effective enrichment through illumination with IR light in a single reactor. Furthermore, the theoretical maximum PHA production capacity achieved by PPB is 0.9 molPHA molAcetate^{-1} (Fradinho *et al.*, 2019) vastly higher than any aerobic process

(Bengtsson *et al.*, 2010), which could potentially increase the volumetric production of PHA.

The illumination requirement might be one of the biggest drawbacks when employing a photoheterotrophic process using PPB. According to recently analyzed data, the illumination costs to produce PPB are 1.68€ kgbiomass^{-1} (Capson-tojo *et al.*, 2020), which would make the process economically unfeasible. Several strategies could be carried out in scaling up the proposed process to reduce illumination costs. The production of PHA has been studied through light / dark cycles, obtaining good results (Fradinho *et al.*, 2013). Thereby, the use of raceway reactors that take advantage of using natural light for PHA production may entail a cost reduction of 40%. Currently, this option is under investigation at a semi-industrial scale within the first photobiorefinery in Europe, constructed in the framework of the BBI-H2020 Deep Purple project focused on the extraction and recovery of high value-added resources with PPB (https://deep-purple.eu/). However, it is possible that PHA productivity would be reduced as well, as the reactor would be in dark mode half of the operative time. On the other hand, if artificial illumination is used, MPBR with cell-retention systems could circumvent the problem of low biomass production. MPBR have been extensively applied in wastewater treatment with PPB (Hülsen *et al.*, 2016), **but little research has been performed on the frame of PHA production.** Another advantage of using this type of reactor is the possibility of the co-production of biohydrogen, which is not possible in open raceways. In any case, recent results have enlarged the economic capabilities of PPB-based technologies, as the minimum irradiation needed for wastewater treatment has been demonstrated to be much lower than suggested before, at around 1.4 W m^{-2}, corresponding to 0.33 kWh m^{-3} or 3.96 kWh m^{-3} d^{-1} (Dalaei *et al.*, 2020). Thereby, a more detailed analysis of the capabilities of natural-irradiated PPB technologies is needed to allow for the up-scaling of the process, which is currently ongoing. In any case, it is key to increase the amount of SCCA in the liquid effluent, in order to make the organic matter more readily available for accumulation in PHA. **Accordingly, in the following sections of this Thesis, the acidogenic fermentation of the hydrolysate after thermal pretreatment will be studied, with the objective of increasing the percentage of SCCA present in the liquid fraction.**

In our opinion economic viability can only be achieved through an integrated biorefinery process, where all process streams are valorized. The anaerobic digestion of the solid fraction is a good complement to achieve a self-

sustained process, but the high C/N ratio of the lignocellulosic biomass is not suitable for a continuous process. Nutrients must be added either through the supplement of a synthetic mixture of nutrients or by mixing with another high nutrient content waste (e.g. domestic sewage or the **OFMSW**) in a co-digestion process. This will not only improve the continuous process but may favor the possibility of using the remaining digestate as an organic fertilizer.

Another possibility to increase the sustainability of the concept relies on the re-use of the remnant COD caused by its partial consumption during photo-heterotrophic treatment. Lack of nutrients caused an important excess of the soluble substrate at the end of the photo-bioprocess. Thereby, an increase of nutrients would theoretically improve the biomass productivity of the process due to the consumption of the excess of SCOD. This in turn can affect everything related to the biomass, including PHA productivity. The co-substrate fed to the anaerobic digestion can also provide nutrients when these are released into the digestate and recirculated into the photobioreactor. Another option to consider is to derive the excess of soluble organic matter for anaerobic digestion since it is a way to ensure the closure of the carbon cycle, important within a circular economy. As commented before, this liquid fraction increases methane production, favoring the viability of the proposed integrated process. All these options must be studied to check their effect on the operational strategy and the capital costs. This biorefinery concept is taking its first steps and there are still big gaps in the knowledge for the scalability of the process. Nevertheless, this is also an opportunity for further research and optimization.

4.I.13. CONCLUSIONS

This study shows for the first time the possibility of using the hydrolysate coming from the thermal hydrolysis of lignocellulosic residues as a substrate to feed PPB for the production of PHA (a peak of 20 wt. % of PHA after a 120 °C has been achieved). Thermal pretreatment improves the organic matter solubilization as well as digestibility of the remaining solids but may also limit PPB growth due to low nutrient release. These nutrients can be sourced externally to enhance the productivity of the concept. The anaerobic digestion of the solid fraction complements the process by producing biogas that serves to achieve energetic autarchy. The proposed PPB integrated biorefinery concept shown in this work offers potential and several alternatives for the reduction of PHA production costs, inviting future research.

4.I.14. REFERENCES

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SECTION II: ACIDOGENIC CO-FERMENTATION

Preamble and rationale

The LW is the least biodegradable part of the OFMSW. According to the latest published data, in Spain, 42% of the MSW is the organic fraction (OFMSW), 20% of which is lignocellulosic pruning and gardening waste. This section aims to settle the possible synergistic effect when co-fermented FW and LW wastes produce SCCA and H_2. For the first time, the work also analyses the effect of a steam explosion pretreatment on the co-fermentation performance. Finally, this work explores several strategies for producing high-value-added products from SCCA within the framework of the EU bioeconomy.

Experimental design

The experiments have been conducted using as substrate the FW and LW described in Chapter 3. The steam explosion pretreatment was performed at 150 °C and 40 min of reaction, since these are the conditions optimized by our research group in the context of the DEEP PURPLE project and the results are currently in preparation. The acidogenic fermentation of FW and LW with mixtures of 20%, 50% and 80% LW by volume of VS, both without and after pretreatment, is studied. The experimental desing conditions are summarized in Table II.1.

Tabla II.1 Summary of the experimental design used in section II.

Variable / Process	Section II
Waste	FW and LW
Thermal hydrolysis	Steam explosion reactions 150 °C and 40 min
Acidogenic fermentation	Thermophilic batch tests of the untreated and pretreated wastes. Mixtures with 20%, 50% and 80% of LW in volume.

4.II.1. COD SOLUBILIZATION AND STEAM EXPLOSION PRETREATMENT

Figure II.1 reports the COD solubilization in the fermentation process. Usually, thermophilic temperatures favor the solubilization of organic matter compared to mesophilic temperatures, as previously observed (Soomro *et al.*, 2020). The solubilization of COD for FW reached 32%. However, the hydrolytic performance in the LW case was significantly lower (14%). The proliferation of hydrolytic microorganisms inherent to food waste and the difference in compositional characteristics of LW and FW may have impacted the hydrolytic microbial populations in batch tests, resulting in reduced hydrolysis of LW (Arras *et al.*, 2019). The co-fermentation processes offer a positive synergy since COD solubilization of approximately 30% is achieved, even for a high percentage of LW (80%), probably due to these different communities.
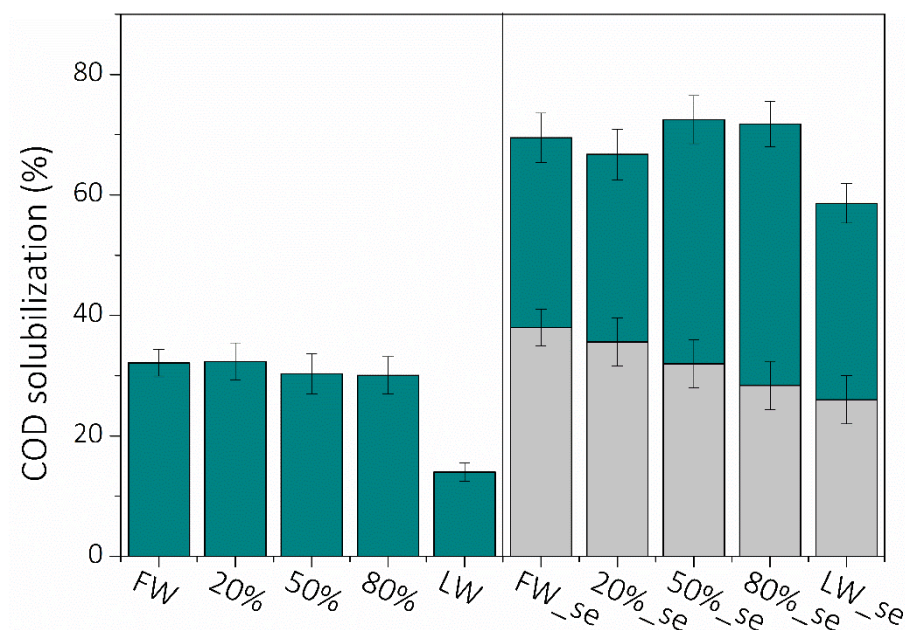

Figure II.1 Effect of steam explosion pretreatment (grey bars) and acidogenic fermentation (dark cyan bars) on COD solubilization for different mixtures of FW and LW.

Soluble COD increases coupling steam explosion pretreatment and acidogenic fermentation. Table II.2 shows the main characteristics of the pretreated substrates as FW_se and LW_se. The decrease in VS mainly indicates that hemicellulose and cellulose were decomposed to lower molecular organics, mostly oligo- and monosaccharides and some organic acids (Bundhoo *et al.*, 2015). Lipids are hydrolyzed into fatty acids through beta-

oxidation (Yin *et al.*, 2014). On the other hand, protein is hardly decomposed during hydrothermal pretreatment (Pavlovič *et al.*, 2013), which agrees with our results. We obtained a 42% TS destruction and 38% COD solubilization of the FW, and a 30% TS destruction and 26% COD solubilization of the LW. However, the total C: N ratio of the FW after pretreatment was 100:3.1, whereas it was 100:1.4 in the liquid phase. This data indicates preferential dissolution of organic carbon instead of nitrogen. A similar pattern occurs after the LW pretreatment, where a total C/N ratio was 100:1.9, but the liquid fraction was 100:1.0. Also, a small amount of SCCA was released, being acetic acid the most abundant, accounting for 72% of the obtained SCCA.

Table II.2 Average and 95% confidence intervals of the macroscopic characteristics of the organic solid waste used in this studio: FW (Food Waste), LW (Lignocellulosic waste), FW_se (Food waste after the steam explosion), LW_se (Lignocellulosic waste after the steam explosion).

	FW	LW	FW_se	LW_se
TS (g kg^{-1})	115 ± 12	950 ± 12	55 ± 14	66 ± 16
VS (g kg^{-1})	99 ± 6	911 ± 11	48 ± 9	75 ± 6
TKN (gN kgTS^{-1})	3.2 ± 0.5	2.5 ± 0.8	3.6 ± 0.9	2.6 ± 1.2
TKN (mgN L^{-1})[a]	150 ± 15		644 ± 22	389 ± 33
TCOD (g L^{-1})	122 ± 5	1075 ± 8**	115 ± 10	136 ± 13
SCOD (g L^{-1})[a]	9.5 ± 0.6		46.2 ± 5.8	38.5 ± 7.5
HAc (% SCOD)[a]			0.5 ± 0.1	2.2 ± 0.3

[a] In the liquid phase **g gTS^{-1}

The pretreatment also improved the solubilization of COD in the fermentation process, with similar solubilizations of around 44% for FW_se and 20%_se and LW_se. For 50%_se and 80%_se, solubilizations of 50% and 53% occurred, respectively, with maximum solubilization through both processes close to 80% of the COD. These results showed a highly efficient combined treatment in COD solubilization, with an intimate link to solids destruction and process waste reduction (Yin *et al.*, 2014).

Another critical factor in the hydrolytic process is the pH, as a decrease of the pH values below 4.5 - 5.0 strongly inhibits the hydrolytic and fermentative bacteria (Moretto *et al.*, 2019). Figure II.2 shows the pH evolution and in almost all cases the pH drop occurs down to 5. We encourage further studies to focus on thermophilic operations under strict pH control to sustain high hydrolysis rates and reduce the fermentation time.
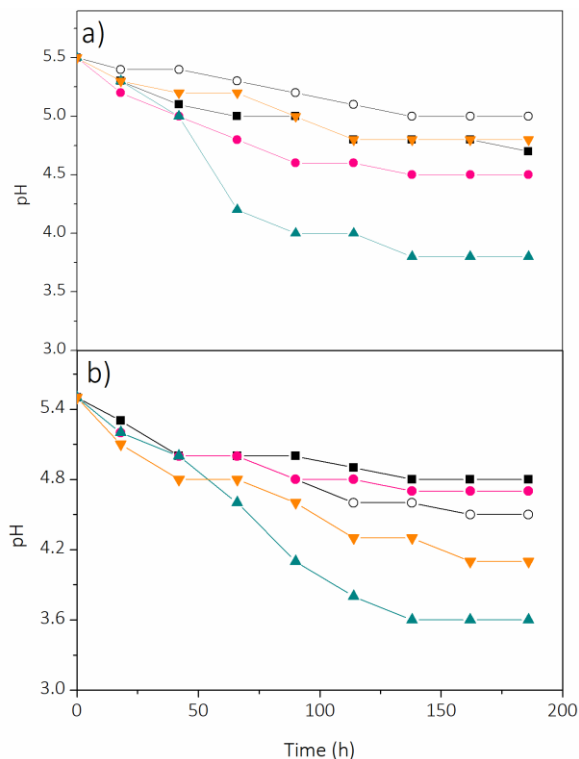
Figure II.2 Change in pH pattern during the batch fermentation using non- pretreated waste (a) and pretreated waste (b). Wastes used were 100% FW (●), 100% LW (○), and mixtures of 20:80% (●), 50:50% (▲) and 80:20% (▼) of FW and LW, respectively.

4.II.2. SHORT CHAIN CARBOXYLIC ACIDS PRODUCTION

Figure II.3 depicts the time course of the released SSCA on a total COD added basis during the acidogenic fermentation. The endogenous production of the inoculum is negligible, but it is still subtracted from the production. Figure II.2a summarizes the results of the SCCA production in acidogenic fermentation when using non-pretreated substrates. Y_{SCCA} obtained with FW is 0.33 ± 0.01 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$, and that of the LW is significantly lower with 0.13 ± 0.01 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$. Data on SCCA production has not been as extensively reported as on hydrogen production. An overview of the available literature shows that the range of transformation into SCCA for wastes of these characteristics, including studies on continuous reactors, is 0.06 to 0.61 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$ (Garcia-Aguirre *et al.*, 2017; Moretto *et al.*, 2019; Soomro *et al.*, 2020; Yin *et al.*, 2014). Considering that this range involves production with substrates that have undergone pretreatment or continuous reactors, the

FW results are substantial. Concerning the different co-fermentations, in this case, a strong positive synergistic effect is observed. The results were 0.33 ± 0.01, 0.24 ± 0.02 and 0.28 ± 0.01 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$ for the 20% 50% and 80% mixtures, respectively. With these data, we can calculate a Y_{SCCA} positive synergistic effect, with an increase in yield with respect to the theoretical of 15%, 5%, and 61%, respectively.
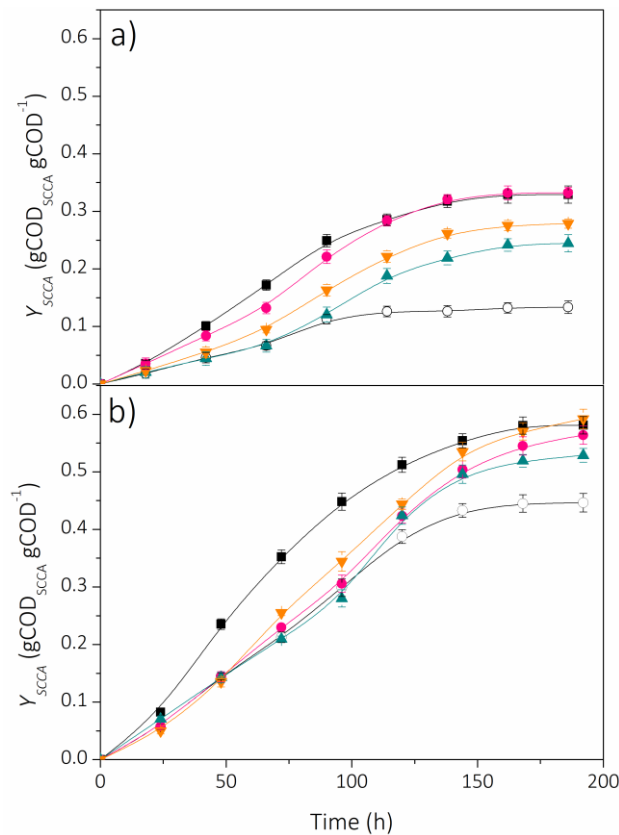

Figure II.3 SCCA production during batch tests on non-pretreated (a) and pretreated wastes (b) at different FW-LW ratios: 100% FW (■), 100% LW (○), and mixtures of 20:80% (●), 50:50% (▲) and 80:20% (▼) of FW and LW, respectively.

Figure II.2b shows the results obtained on SCCA production using pretreated substrates. The Y_{SCCA} obtained for FW_se was 0.56 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$, representing a 70% increase compared to the untreated sample. This result is among the highest in the literature range, and considering that these are batch tests, there is room for improvement in continuous mode as previously demonstrated, for example, in Yin *et al.*, (2016). In the article by Yin *et al.* (2014), the increase was 42.5% in batch-type tests. Although the result

obtained for LW_se is the lowest (Y_{SCCA}: 0.44 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$), this represents an increase of 215% compared to the same waste without pretreatment. Again, minimal research has been done on SCCA production with this substrate when combining steam explosion pretreatment with fermentation. For example, in the article by Perimenis *et al.* (2016), they reported very low productions on substrates with high lignin and cellulose content such as wheat bran (0.17 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$) or on miscanthus (0.11 gCOD$_{SCCA}$ g COD$_{added}^{-1}$), but even worse results (0.17 vs. 0.22 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$) on wheat bran. The co-fermentation achieved the highest yields in our work, with 0.58 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$ for the 80%_se mixture and 0.56 and 0.57 gCOD$_{SCCA}$ gCOD$_{added}^{-1}$ for the 20%_se and 50%_se mixtures, respectively. This result translates to a positive synergistic effect and an increase in Y_{SCCA} of 5%, 6%, and 8%, respectively. Although the synergistic effect was clear without pretreatment, the overall performance in SCCA production was significantly superior after pretreatment by steam explosion.

4.II.3. PROCESS EFFICIENCY: HYDROLYSIS EXTENT, ACIDIFICATION, AND SYNERGY EFFECT

Figure II.4 shows the variations in the acidification extent, the hydrolysis extent (H_E), and the synergy effect (φ). The H_E is assimilable to the efficiency of the process. It represents the particulate COD solubilized and hydrolyzed during the fermentation by the hydrolytic bacteria and the hydrogen produced from that SCOD. COD solubilization is far more relevant in establishing this parameter than hydrogen production since the COD$_{H2}$ in these fermentation trials ranged from 0.9 to 2.1% of the initial total COD. Without pretreatment, the maximum H_E achieved was 38%, observed with FW. This result fairly agrees with others reported in the literature, ranging from 31 to 40% (Soomro *et al.*, 2020). Furthermore, the LW got the lowest H_E with 19%, which concurs with the more serious difficulty of hydrolyzing lignocellulosic wastes.

Co-fermentations yielded very positive synergistic effects (φ), the highest being 1.53 for the 80:20 FW/LW mix ratio and 1.12 and 1.27 for the 20:80 and 50:50 mix ratios, respectively. These results confirm the possibility of using this technology with the usual mixtures obtained from municipal solid waste. The use of lignocellulosic wastes as co-substrate considerably improved the efficiency of the process compared to the treatment of single substrates. This type of synergy was also observed, for example, in the co-fermentation of MSW

and LW for bioethanol production, where synergistic effects of between 1.10 and 1.49 were achieved (Zhang *et al.*, 2020). Also, Soomro *et al.* (2020) reported a synergistic effect between synthetic food and paper waste, with synergies ranging between 1.08 and 1.66. Considering that the substrates used in this study are urban wastes, pruning LW is much less biodegradable than paper waste, and the batch nature of our tests, the synergistic effect of our results is highly remarkable.
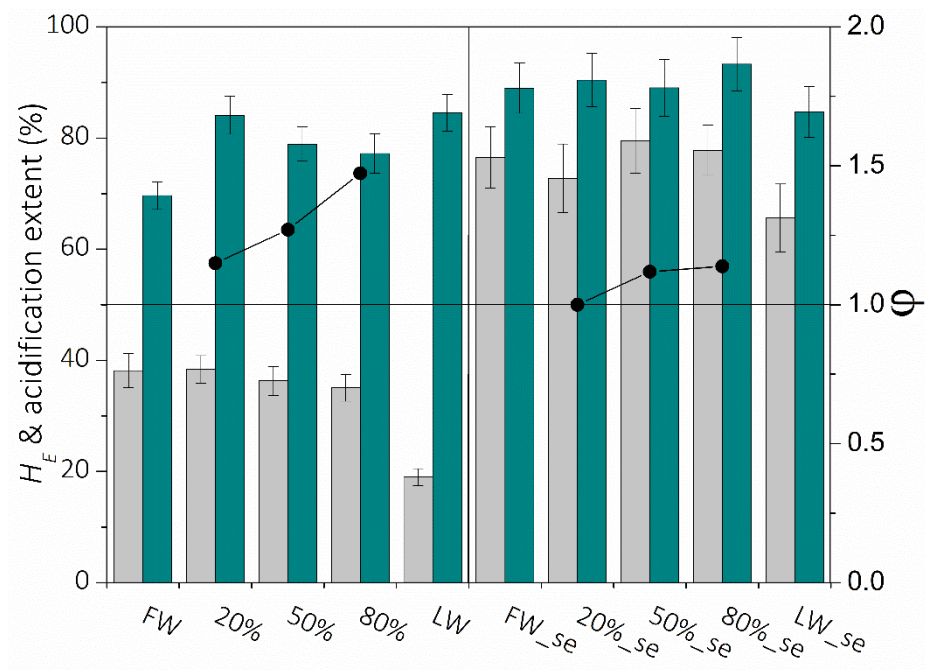

Figure II.4 Overall productivities of the fermentation process. Grey bars represent hydrolysis extent (H_E), and dark cyan bars show acidification degrees (AD). Black dots show the synergy index (φ).

On the other hand, as shown previously, the steam explosion pretreatment considerably improved the fermentation efficiency, using both single and mixtures of urban waste. The H_E reached up to 72% and 65% on single FW and LW fermentation, respectively, representing a 100% and 245% increase compared to the non-pretreated process. But co-fermentations reached the highest H_E, achieving the following results: 72, 79, and 77% for 20%_se, 50%_Se, and 80%_se, respectively. In this case, the synergies obtained were slightly lower than those found in the non-pretreated experiments, with φ

values of 1, 1.12, and 1.15, respectively. Again, these results confirm the suitability of mixing these wastes within an acidogenic fermentation process.

The acidification obtained without pretreatment are in the range of 70-85%. In this case, the highest acidification range was for the LW, undoubtedly due to the soluble protein and carbohydrates still present in the FW. For this parameter, we found a wide range of results in the literature, from 48% to 94% (Moretto *et al.*, 2019; Perimenis *et al.*, 2016). This study also showed an increase in the FW_se from 69% to 89%. However, in the literature, we found that thermal pretreatments can be detrimental to this parameter. Yin *et al.* (2014), reported that the acidification range decreases from 80% to 70% after a thermal pretreatment at 140ºC. On the other hand, in the LW_se, acidification does not vary, keeping it at 85%. Previous studies with lignocellulosic residues showed a slight increase when miscanthus was used (from 79 to 83%) or even a decrease with wheat bran (from 65 to 55%) (Perimenis *et al.*, 2016)v. In this case, the different co-fermentation mixtures obtained the highest acidification range values with 90, 89, and 93% for 20, 50, and 80%, respectively, again demonstrating the improvement of the process caused by mixing these wastes.

4.I.4. SCCA COMPOSITION AND HYDROGEN PRODUCTION

Figure II.5a shows the distribution of SCC for the different non thermally pretreated wastes. The features of the waste stream can change the microbial community and change the dominant species in the anaerobic digestion process (Garcia-Aguirre *et al.*, 2017). As shown in Figure 5.4a, when the substrate is mainly composed of FW, acetic acid is the primary fermentation product (up to 44%), and lactic acid production is close to 6%. Indeed, acetogenic fermentation is the most common pathway during FW fermentation due to the equilibrated composition in carbohydrates, lipids, and proteins (Yin *et al.*, 2014). On the other hand, the LW drifted to butyric fermentation (up to 43% butyric acid), and no lactic acid appeared. Butyric fermentation is also typical when dealing with LW residues, mainly composed of sugars and oligomers (Perimenis *et al.*, 2016). Also, LW causes an increase in propionic acid production (14% compared to 10%) but a slight reduction in valeric and isovaleric acid compared to FW. Both urban wastes yielded up to 5% hexanoic acid, and oddly, no iso-butyric acid production was observed in almost all conditions. Other carboxylic acids found in small traces were caproic, oxalacetic, succinic, or fumaric acid. The maximum observed was 2% caproic

acid obtained with LW. However, the prominent exception occurred in the 50:50 mix condition, where a high generation of lactic acid happened. This lactic acid production is consistent in triplicates and reaches up to 28% prominence, which required further analysis that was possible after checking the hydrogen production data.
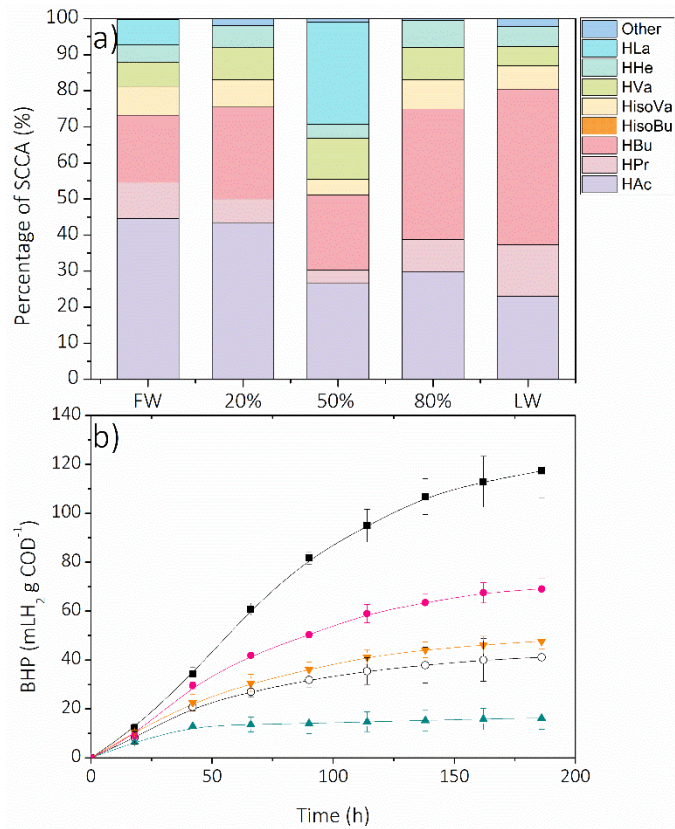

Figure II.5 Distribution of SCCA at the end of batch tests using non-pretreated waste sources (a) and associated H_2 production (b) at different FW-LW mix ratios. FW (●), LW (○), and mixtures of 20% (●), 50% (▲) and 80% (▼). HAc: Acetic acid, HPr: Propanoic Acid, HBu: Butiric acid, HisoVa: Isovaleric acid, HVa: Valeric acid, HHe, Hexanoic acid and HLa: Lactic acid.

Figure II.5b shows hydrogen production from the batch tests with non-pretreated single and mixed substrates. Hydrogen was produced successfully with both substrates, although production is much higher with FW than with LW. Hydrogen yields were 117 ± 11 and 41 ± 8 mlH_2 $gCOD^{-1}$ added for the FW and LW experiments, respectively. The mixtures produced 69 ± 4, 16 ± 4 and 47 ± 3 mlH_2 $gCOD^{-1}$ for 20:80, 50:50 and 80:20 FW/LW mix ratios, respectively. The literature reported highly variable values of hydrogen production from the

urban wastes, ranging from 12 - 248 mlH$_2$ gCOD^{-1} (Basak *et al.*, 2020; Bundhoo *et al.*, 2015; Kuang *et al.*, 2020). This variability is mainly due to the heterogeneity of the substrate and the different technologies and pretreatments used. Our results fall in the middle of this range. However, these are significant results considering that neither the substrates nor the inoculums were pretreated.

Figure II.6a shows the analysis of the fermentation products coming from the pretreated mixtures. The fermentation of FW is still driven to acetic acid (36%) but at a lower percentage than without pretreatment. In addition, the fermentation of FW promoted the production of lactic acid (23%). Again, the fermentation of LW fostered butyric acid production, although in lower values (up to 33%) than without pretreatment. LW_se also produces lactic acid, up to 5%, which was much lower than in FW. Indeed, the mixtures promoted the production of lactic acid when the proportion of FW increased. In contrast, when the ratio of LW increased, the fermentation products trend towards propionic acid (8% -12%), valeric acid (2% - 8%), and isovaleric acid (2.7 - 13.5%), to clear detriment of the lactic acid production. Besides, isobutyric acid appeared in all conditions, which differed from the tests without pretreatment.

Figure II.6 shows the time course of the hydrogen production during these experiments. Hydrogen production on the pretreated substrates increased considerably. A 28% increase in hydrogen production after the steam explosion was achieved for FW. This increase is slightly higher than typical values in the literature where hydrogen yield improved by 5–20 times compared to the control (Bundhoo *et al.*, 2015). For the pretreated lignocellulosic residue LW_se, an increase of 260% was obtained. The highest increase found in the literature is with an ultrasonic pretreatment with dilute HCl, where a 311% increase in hydrogen production is obtained (Datar *et al.*, 2007). As for the co-fermentation mixtures, the results obtained were as follows: 152 ± 2, 109 ± 4, and 136 ± 2 mlH$_2$ gCOD^{-1} for 20%_se, 50%_se, and 80%_se, respectively, which indicates a reduction in hydrogen production in the co-fermentations, except for 20%_se. Overall, hydrogen yields after pretreatment are higher than without pretreatment, and differences between substrates are reduced.
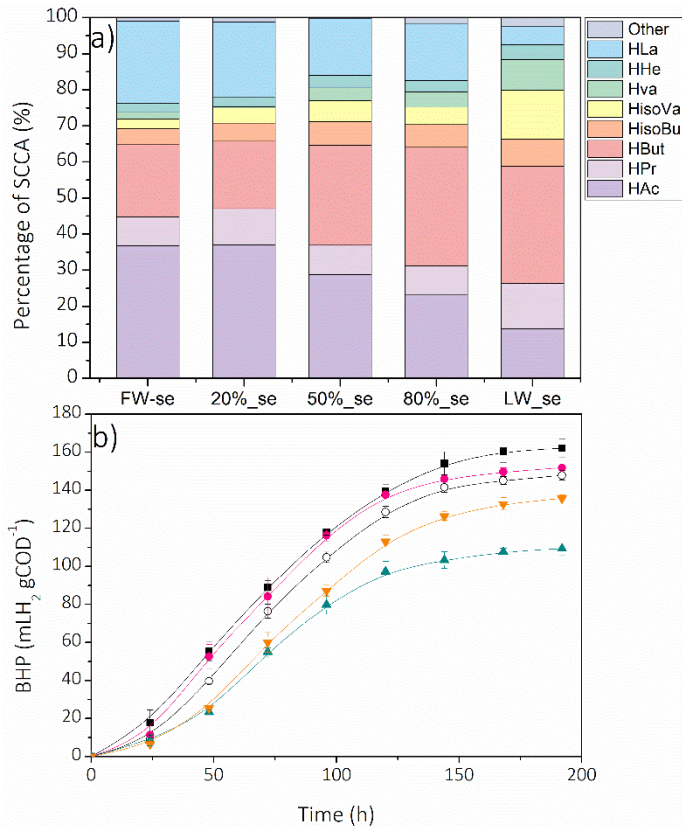
Figure II.6 Distribution of SCCA at the end of batch tests using pretreated waste sources (a) and associated H_2 production (b) at different FW-LW mix ratios. FW (●), LW (○), and mixtures of 20% (●), 50% (▲) and 80% (▼).HAc: Acetic acid, HPr: Propanoic Acid, HBu: Butiric acid, HisoBut: Isobutiric acid, HisoVa: Isovaleric acid, HVa: Valeric acid, HHe, Hexanoic acid and HLa: Lactic acid.

Based on our results, the predominance of acetic acid formation links with a high hydrogen yield, which agrees with the available literature (Hoelzle *et al.*, 2014). Likewise, the butyric acid pathway can produce an excess of acetate as the main metabolites, yielding high hydrogen production. The third most-produced metabolite in almost all cases is lactic acid, but its production tends to increase when there is more presence of LW. On the other hand, when the substrate is LW, the production of valeric, iso-valeric, and propionic acid is increased. This is to be expected, since each substrate enters the fermentation metabolism at a different point, and therefore has a unique set of pathways and yields available to it. The SCCA production profiles and the higher concentration of lactic acid in FW may be due to its higher starch concentration or to the naturally-occurrent presence of lactic acid-producing microorganisms

in FW and is similar to the profile obtained in previous studies (Vidal-Antich *et al.*, 2021).

Low hydrogen yields are associated with propionic acid and reduced end-products such as alcohols and lactate (Ochoa *et al.*, 2021). In our experiments on substrates without pretreatment, the production of lactic acid results in a sudden drop in pH on the 50% ratio test as can be seen in Figure II.2, which appears to be inhibiting hydrogen production since an excess of electrons that may be preventing the acetogenesis process. This mixture of FW and LW contains a high concentration of sugars but also nutrients, and therefore the excess of electrons due to the consumption of sugars leads to lactic acid production, which in turn leads to a decrease in hydrogen production, as explained in Hoelzle *et al.*, (2014).

In the acidogenic fermentation with mixed microbial cultures, lactate and propionate accumulation are viewed as evidence of inhibition within the methanogenic process and are often associated with shock loads of substrate (Hoelzle *et al.*, 2021). Our results point towards that explanation, where the steam explosion pretreatment makes the organic matter much more accessible, thus increasing lactic and propionic acid production. The hydrolysis step is not quite as constraining, and the bacteria are overloaded. They can divert some of the excess electrons to the production of lactic acid, propionic acid, butyric acid, and to a lesser extent other higher molecular weight SCCA, limiting hydrogen production.

4.II.5. Implications and perspectives

A total SCCA concentration up to 0.58 $gCOD_{SCCA}$ $gTCOD^{-1}$ with a high acidification rate as herein obtained is quite promising. In this study, we have shown that, depending on the percentage of lignocellulosic residue that we have in the urban waste, we can anticipate the type of carboxylic acids produced. This fact is essential because, depending on the molecular weight and the chemical structure of the acids, their price varies considerably. For example, caproic and valeric acids have the highest market prices (3800 € $tonne^{-1}$) (Ramos-Suarez *et al.*, 2021), followed by propionic acid (2000–2500 € $tonne^{-1}$), butyric (1500–1650 € $tonne^{-1}$) (Atasoy *et al.*, 2018), lactic acid (550–1950 € $tonne^{-1}$, depending on the lactic acid grade) (Manandhar and Shah, 2020), and acetic acid (400–800 € $tonne^{-1}$) (Atasoy *et al.*, 2018). SCCA are

fundamental building blocks of chemicals such as esters, ketones, aldehydes, alcohols, and alkanes (Greses *et al.*, 2020). But for this purpose, the different acids must be separated and purified, which may not be cost-effective. The work of Bonk and Schmidt, (2015) indicated that SCCA obtained from dark fermentation would be cost-effective if the operating costs of the separation/purification did not exceed 15 $ m^{-3} effluent. To bypass this bottleneck, direct usage of the fermentation products in other bioprocesses is a promising alternative, wherein in any case, some physical processes like centrifugation allow the separation of solid and liquid streams. These bioprocesses entail the production of hydrogen, high added-value products like PHA and biodiesel, chain elongation products, or the removal of nitrogen and phosphorus from wastewater (Ramos-Suarez *et al.*, 2021). Biogas production from the fermentation products is not desirable as recent studies indicated that the generation of SCCA is more profitable than the more traditional AD with biogas upgrading (Bastidas-Oyanedel and Schmidt, 2018). In any case, the AD is useful to produce energy from the degradation of the solid streams upon physical separation of the fermentate, thus making the overall biorefinery process energetically more sustainable (Righetti *et al.*, 2020).

Generally, steam reforming produces massive amounts of hydrogen from non-renewable hydrocarbons, causing high greenhouse gas emissions. In the last years, research efforts focused on developing biotechnological routes to produce hydrogen in an environmentally friendly manner through acidogenic fermentation and photochemical fermentation, even in a single stage (Shiladitya *et al.*, 2017). In this platform, the synergy of both biological processes is the crucial point, where phototrophic bacteria can produce hydrogen with a conversion efficiency of up to 0.98 gCOD$_{H2}$ gCOD$_{fed}^{-1}$ (Puyol *et al.*, 2019). This platform is sui*table* for low nitrogen-bearing feedstock, like lignocellulosic hydrolysates.

A promising bioprocess is chain elongation, which appeared around ten years ago. It mainly consists of converting SCCA into medium-chain carboxylic acids such as caproic acid, enanthic acid, caprylic acid, or pelargonic acid, which, as mentioned before, have a higher market value. This process occurs by β-reverse oxidation, where an electron donor (mainly acetyl-CoA and ethanol) is oxidized to reduce an electron acceptor, e.g., acetic acid (Magdalena *et al.*, 2020). Although most studies on this technology have focused on synthetic media, upscaling chain elongation technologies are underway

(Candry and Ganigué, 2021) and could be an important biotechnological pillar for the production of high value-added products from waste.

Another common application of SCCA is the biological nutrient (nitrogen and phosphorous) removal from wastewaters. Even removing P seems to be more effective by using SCCA from acidogenic fermentation than the traditional use of synthetic acetic acid. In this respect, Tong and Chen (2007) observed that the phosphorus removal efficiency was around 98% with the fermentative SCCA and about 71% using pure acetic acid. Also, several studies have demonstrated that waste-derived SCCA outperforms commercial chemicals in terms of nitrate removal efficiency and denitrification rate when used as a carbon source (Tong and Chen, 2007). Therefore, integrating SCCA production in wastewater treatment plants offers a bioeconomic alternative to removing nutrients.

One of the most promising applications for the SCCA obtained from acidogenic fermentation is PHA production, and the most common process used is sequential enrichment and accumulation steps using aerobic mixed cultures. In this sense, Bengtsson *et al.* (2017) reported an accumulation of up to 49% PHA of volatile suspended solids using SCCA from fermented organic residues as substrates in a pilot-scale demonstration (Bengtsson *et al.*, 2017). However, this technology is still looking for its breakthrough to be economically viable since PHA is not yet competitive compared to equivalent petrochemical plastics such as polyethylene terephthalate (Ramos-Suarez *et al.*, 2021).

Finally, PPB are perfect candidates for PHA production since they can obtain energy from IR light instead of oxygen, reducing operating costs. **In this Thesis, the feasibility of co-producing PHA and hydrogen from the organic fraction of municipal solid waste and lignocellulosic waste has already been demonstrated. However, several challenges remain and will be addressed in the next section of this Thesis.**

4.II.6. CONCLUSIONS

A significant synergy was found between the two most common substrates found in municipal solid waste (FW and LW) in the production of SCCA but not in the production of H_2. Also, the steam explosion pretreatment significantly increases the process yield with a maximum of 0.58 $gCOD_{SCCA}$ $gCOD^{-1}$ and a 93% acidification rate. The results obtained in this study indicate the robustness of

the acidogenic fermentation technology. They can contribute to the valorization of municipal solid as the first step of a biorefinery platform dedicated to recovering added-value bioproducts (such as PHA) through a combined anaerobic multi-step process.

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SECTION III: INTEGRATED PHOTOBIOREFINERY

Preamble and rationale

Having tested the feasibility of growing PHA-accumulating PPB from OFMSW hydrolysates and optimizing both the thermal pretreatment and the acidogenic fermentation process, this Section aims to optimize PHA accumulation in a PPB enriched mixed microbial culture and explore how its production is determined via organic overloading. Furthermore, this study has been carried out in a **continuous novel membrane photobioreactor (MPBR)** using for the first time an OFMSW eluent pre-treated by steam explosion and fermentation as substrate. Finally, the holistic valorization of organic waste was studied in the integrated photobiorefinery proposed to understand the overall impact of this multistrategy approach.

Experimental design

Table III.1 summarizes the most important experimental design conditions. The waste used as substrate was OFMSW, described in Chapter 3. The steam explosion pretreatment was carried out at 150 °C and a reaction time of 40 min. The conditions chosen for the steam explosion were based on previously performed optimization experiments carried out in the context of the DEEP PURPLE project as explain in the previous Section.

Tabla III.1 Summary of the experimental design used in section III.

Variable / Process	Section III
Waste	OFMSW
Thermal hydrolysis	Steam explosion at 150 °C and 40 min.
Acidogenic fermentation	Continuous treatment of the pretreated waste on a CSTR at thermophilic temperature and pH 5.5
Photoheterotrophic process	Continuous treatment of the liquid fraction of the fermentate MPBR.
Anaerobic digestion	BMP batch tests of the solid fraction accumulated of the fermentate

The hydrolysate obtained after the steam explosion is used directly as feed for an acidogenic CSTR fermenter reactor. An OLR of 4 gCOD L^{-1} d^{-1} and a HRT

of 5 d were fixed and kept for 98 d. These operating conditions were chosen to give operational continuity to the adapted inoculum fed with AFM over a two year period, as explained in Chapter 3. The fermentate was centrifuged to separate the solids from the liquid phase. Next, the solids are fed to an BMP tests to determine its the methanogenic potential, and the liquid phase is fed to the continuous photoheterotrophic process in a MPBR.

The MPBR operation conditions (OLR and SRT) were changed to increase PHA productivity. During the first five days of operation, the MPBR was fed with MOM for acclimatization (described in Chapter 3). Afterward, the liquid fraction of the fermenter effluent was fed, and the operating conditions were varied in 7 Stages as can be seen in Figure III.1, which can be classified as Start-up (S0), operation under stable biomass growth (S1 and S2), first carbon overload (S3), biomass recovery (S4 and S5) and second carbon overload (S6). Due to extreme weather conditions (snowstorm), access to the laboratory was not possible and thus reactor sampling was not performed from day 54 to 60. Representative samples were chosen from the MPBR twice a week for the analysis of the development of the bacterial communities, ensuring that each phase studied had at least three samples each, except for the S0 acclimation phase, where only one sample was collected.
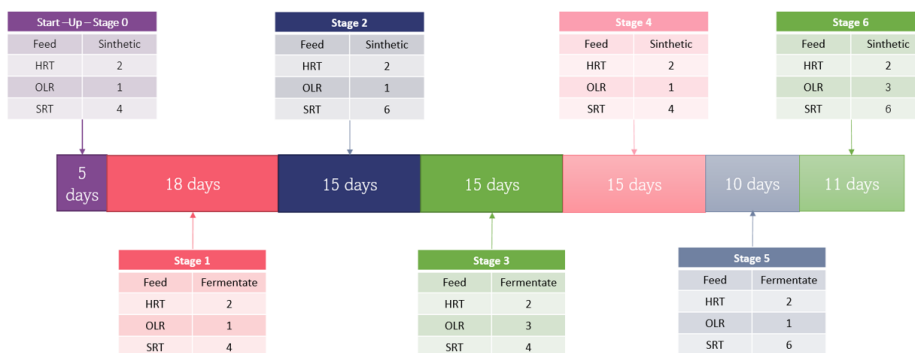

Figure III.1 Schematic timeline of changes in the operating conditions of the MPBR.

4.III.1. STEAM EXPLOSION AND ACIDOGENIC FERMENTATION PRETREATMENTS

The steam explosion disruption apparatus and experimental conditions provided reproducible conditions between the different batches performed. Figure III.2 shows the release of soluble organic matter with a final result of 40% soluble COD on average. Considering that the soluble COD before the steam explosion was 12%, there is an increase in soluble COD of 43%. An average of 7% SCCA is measured, mainly HAc, but also HBu and HPr. The majority of soluble COD is unidentified organic matter (33%), while the remainder is particulate COD. This pretreatment reproducibly solubilizes a substantial percentage of the COD present in the initial waste. This solubilization is essential to enhance the hydrolysis rate, a limiting factor in most biological processes, including acidogenic fermentation.
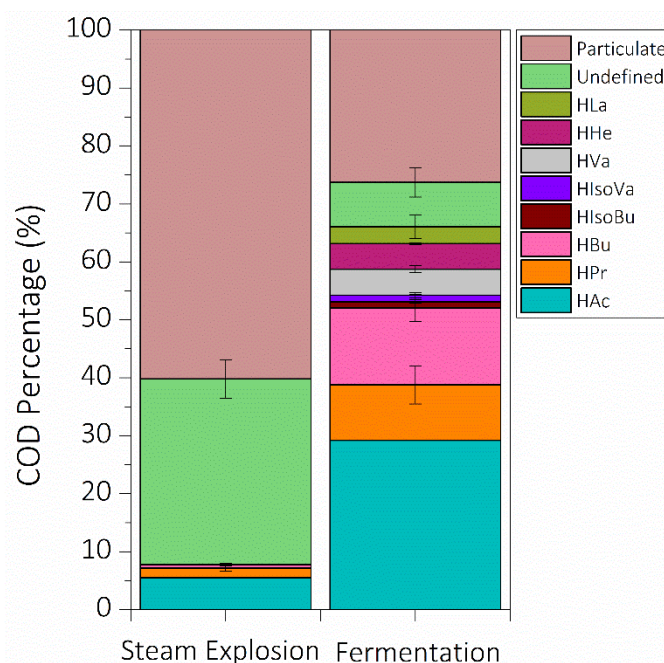
Figure I.2 Steam explosion (4 batches) and subsequent acidogenic fermentation (average of 70 d, after reaching steady-state) in terms of COD mass balance.

A thermophilic acidogenic fermentation was carried out for nearly 100 d as a complementary pretreatment. This process significantly increases the soluble COD, especially the percentage of SCCA produced (Figure III.1). Soluble COD increased up to 74% of the total COD. A total of 65.6% of all COD was transformed into SCCA, which represents acidification of the process of 90%.

HAc and HBu are the two major acids produced, accounting for 63% of the COD equivalent of all SCCA. If we compare these results with the batch results of Section II, we can observe that we obtain a similar acidification (close to 90%) but a 13% higher SCCA production, as expected for a continuous operation. Figure III.3a shows a stabilized yield of about 0.66 gCOD$_{SCCA}$ gCODfeed^{-1}. Combining both pretreatments on the OFMSW to produce SCCA achieves high yields and high stability over time. In addition, H$_2$ is co-produced at high yields during the acidogenic fermentation process.
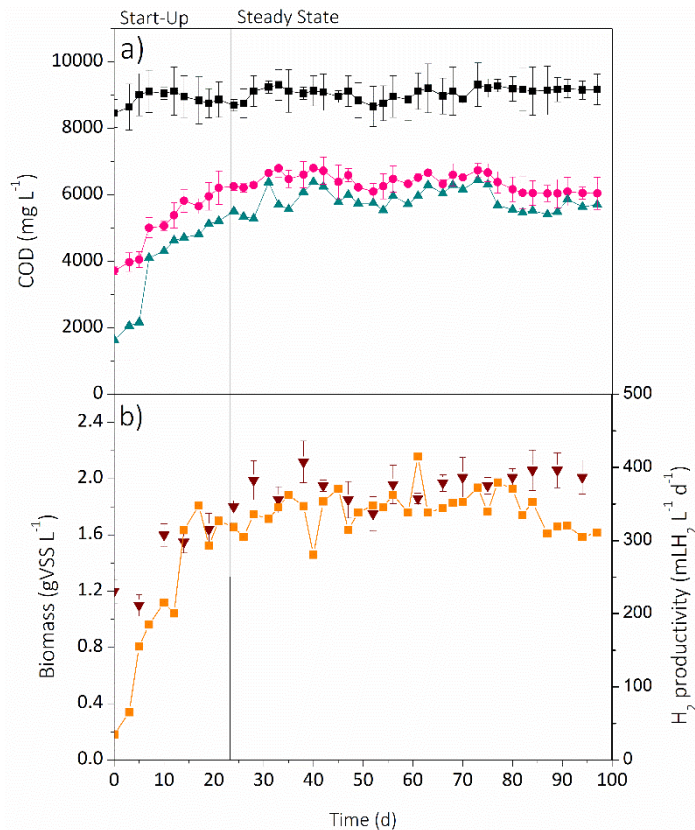
Figure III.3 Acidogenic fermentation reactor operation profile for 98 d. a) Shows TCOD (■), SCOD (●) and COD equivalent of all SCCA (▲). B) shows H$_2$ productivity (■) and biomass (▼) within in the reactor (b).

Figure III.3b shows the hydrogen production and VSS amount present in the fermenter. The stabilization of both parameters is similar to the COD and H$_2$, which could serve as an online indicator of fermentation performance. Average H$_2$ productivity was 345 mLH$_2$ L^{-1} d^{-1}, with a hydrogen percentage of 57% in the outlet gas (43% CO2), and stabilized biomass close to 1.8 gVSS L^{-1}. In any case,

based on the total COD balance, H_2 production never exceeded 3% of the total COD equivalent, confirming that it is a high-value co-product, but represents a small percentage of the total organic matter in the OFMSW valorization.

4.III.2. THE PHOTOHETEROTROPHIC TREATMENT OF THE LIQUID FRACTION WITH PPB

Start-up (S0)

Figure III.4 shows the evolution of biomass, COD removal, PHA (Y_{PHA}), glycogen (Y_{GLY}) and EPS (Y_{EPS}) accumulation and H_2 production for the different scenarios. The culture underwent an adaptation period during the first 5 d (Stage 0) to promote purple bacteria's growth using a synthetic substrate. The biomass went through an exponential growth phase from the second to the third day and increased to 1.5 gVSS L^{-1}, with COD consumption reaching 85%. The correct acclimatization is corroborated through bacterial community analysis with a sample from day 4 (D4).

Figure III.5 shows a culture highly enriched in PPB (>80%). The most prominent genus was *Rhodopseudomonas* sp. with more than 60% relative abundance, followed by *Rhodobacter* sp. with 15%. *Rhodovibrio* sp., *Rhodopila* sp., and *Rubrivivax* sp. were also identified with abundances lower than 1.2%. Glycogen was not measured in the first 4 d of acclimatization, and the average percentage of PHA accumulation was 5% in dry mass, mainly composed of PHB monomer (98% PHB and 2% PHV). From the beginning of the operation, NH_4^+ consumption is complete (Figure III.6), but no H_2 production is detected. The acclimatization Stage was considered finished on day 5 when COD removal efficiency stabilized, and hydrogen production started. Then, the liquid fraction of the fermentate was fed as a substrate.
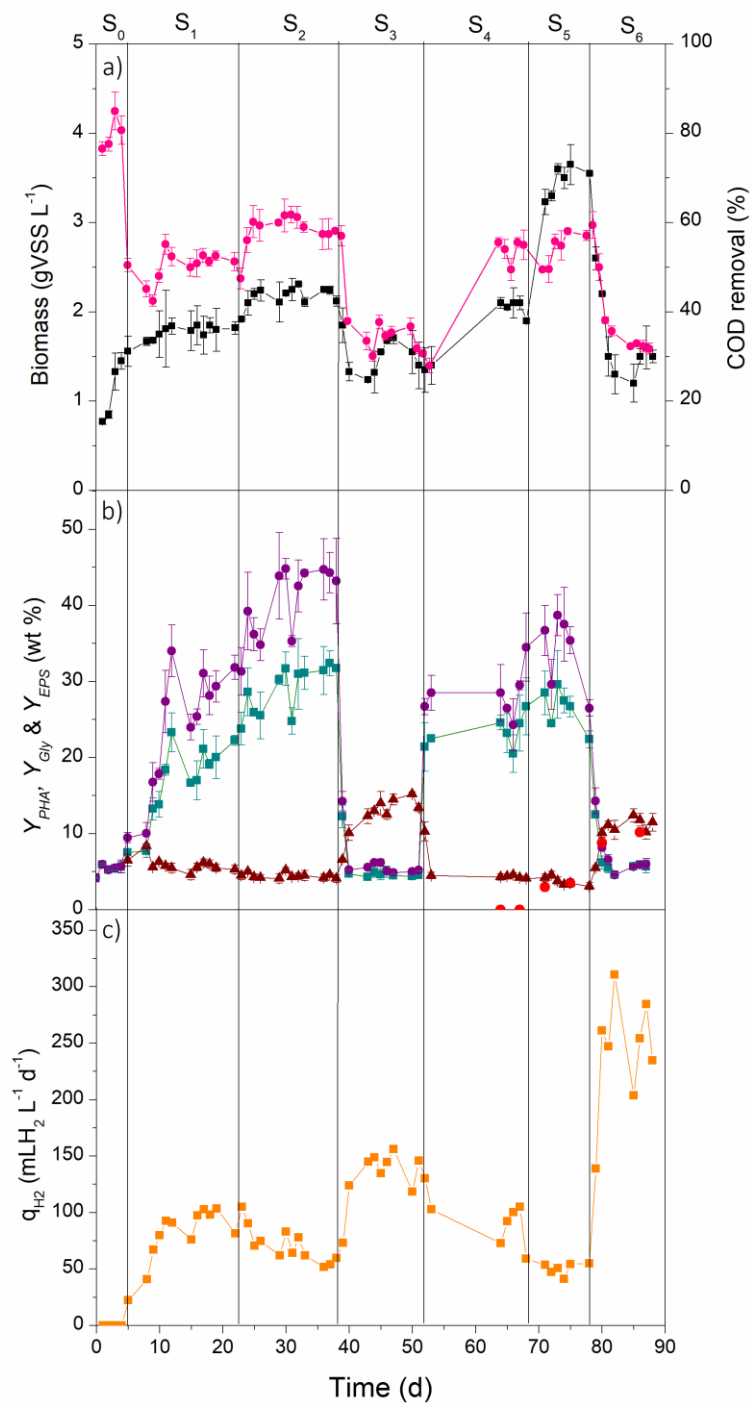
Figure III.4. MPBR operation profile for 88 d. a) shows biomass (■) and COD removal (●). b) shows PHB/PHV percentage (■) and total percentage of PHA (PHB/PHV/PHH) (●), represent glycogen percentage (▲) and represent EPS percentage (●). c) Shows hydrogen productivity (■).
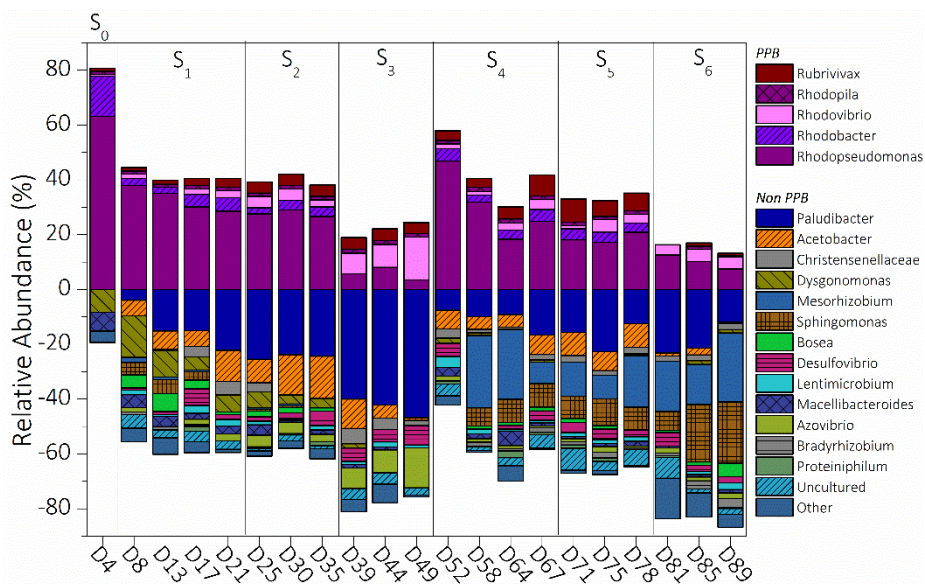
Figure III.5 Microbial community structure at genus level MPBR at different phases.

Operation under stable biomass growth (S1, S2)

Stable biomass growth and high PHA accumulations were obtained in Stages 1 and 2. The low organic load and a substrate composed mainly of SCCA but with limited nutrients first favored the growth of biomass and PHA accumulation, as shown in Figure III.4. In Stage 1, COD consumption decreased compared to Stage 0, to an average of 50%. However, biomass continues to grow to an average of 1.7 gVSS L^{-1}. This COD consumption was limited by the availability of nutrients (Figure III.6). PHA accumulation increased significantly, and then it stabilized at around 31% dry weight during the following 12 d. This PHA comprised PHB, PHV (20% and 5% by dry weight), and PHH that was also detected, accounting for 6% by dry weight. Average production of 6% glycogen was also observed in a very stable pattern. Interestingly, hydrogen evolved with a similar pattern to that of PHA.

In Stage 2, the SRT increased the sludge age, thus keeping the biomass longer in the reactor. In this Stage, the biomass concentration slightly increased to 2.2 gVSS L^{-1} while the COD consumption rose to 60%. PHA accumulation took 4 d to stabilize at an average of 44%, with PHH being about 30% of total PHA by weight. Glycogen accumulation slightly dropped to 5% by weight. Hydrogen, unlike Stage 1, seems to have an inverse relationship with PHA accumulation. As expected, upon adding the liquid fraction of the fermentate, the pressure of

cross-contamination on the culture increased, and the relative abundance of PPB dropped to just over 40%, with *Rhodopseudomonas sp*. being still the predominant genus (Figure III.5). In both Stages, a stable PPB-enriched culture was achieved, with high PHA accumulations and hydrogen yields, with inverse trends. Given these promising results, the organic load was increased to examine possible alterations in the metabolic pathways of PHA accumulation.
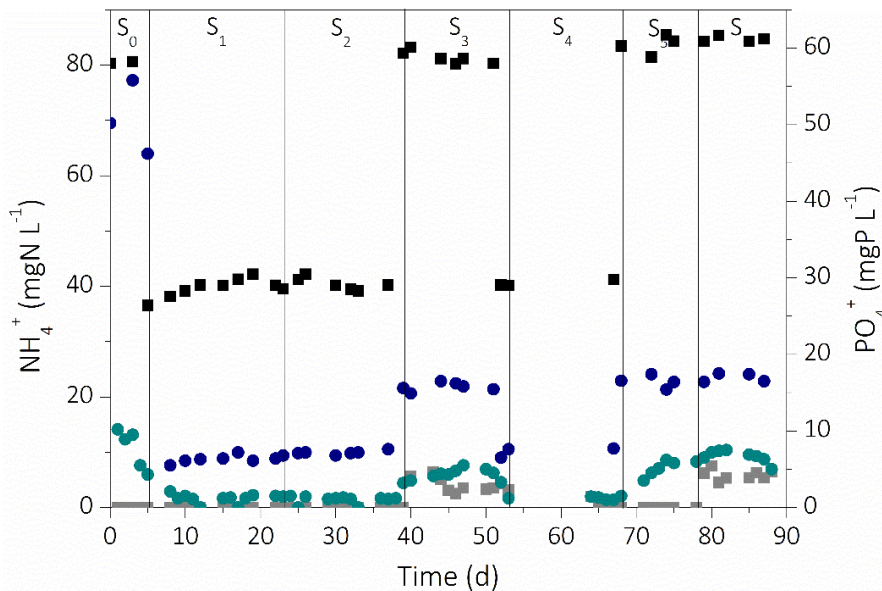
Figure III.6 Nutrient profile during the MBPr operation. NH_4^+ (■) and PO_4^+ (●) in the reactor feed. NH_4^+ (■) and PO_4^+ (●) at the reactor outlet.

First carbon overload (S3)

In Stage 3, the OLR was increased up to 3 gCOD L^{-1} d^{-1} to analyze the culture behavior under an overload episode. The culture immediately fails to accumulate PHA. However, glycogen accumulation and hydrogen production increased. As shown in Figure III.4, biomass collapsed, reducing to 1.3 gVSS L^{-1}, although after 5 d, it stabilized at 1.6 gVSS L^{-1}. The COD consumption fell to 36%, though the limitation of COD consumption was not driven by nutrient availability, as both NH_4^+ and PO_4^+ were not 100% consumed, as shown in Table III.1. The most drastic reduction was PHA accumulation, reduced to 5%. This minimal accumulation remained stable and did not increase during the 14-d phase. As PHA accumulation dropped, glycogen increased to 12%, and hydrogen production doubled. In addition, a decrease in PPB to less than 20% was observed, with *Rhodovibrio sp.* unusually increasing to 15% (Figure III.5).

The next step would be to recover the biomass and a more PPB-enriched culture, thereby studying whether PHA accumulation is regained backing to the conditions of Stage 1.

Recovery of the biomass (S4, S5)

Under the operation conditions of Stage 1, the culture gradually recovered its performance, demonstrating the resilience of this technology. In Stage 4, biomass stabilizes at 1.8 gVSS L^{-1}, and COD consumption is up to 55%. The PHA accumulation was recovered, achieving 28% dry weight (5% PHH), and glycogen again lowered to 5%. The hydrogen production trend was also negative, decreasing its productivity. In Stage 5, the SRT was increased and the same trend as Stage 2 was observed, increasing the biomass concentration considerably up to a maximum of 3.6 gVSS L^{-1} and slightly the PHA concentration. In both Stages, the percentage of PHH is slightly reduced compared to Stage 3, with an average of 25% of the total PHA dry mass. Glycogen accumulation and hydrogen production follow decreasing trends, as in Stage 4. Since Stage 3 showed slight granulation in the culture (Figure III.7), EPS were measured to see if the culture was producing them. At Stage 4, the culture did not show as much granulation, and no EPS was detected. However, at Stage 5, EPS reached 5% dry mass. As for the communities, up to 55% abundance of PPB was recovered in Stage 4 (Figure III.5) but stabilized at around 40% in Stage 5, with the only difference being a higher abundance of Rubrivivax sp. In short, biomass could be restored, significantly increasing its concentration and PHA accumulation, while glycogen accumulation and hydrogen production decreased again. We proceeded again to perform an organic overload to see if the tendencies observed were reproducible in the last Stage.

Second carbon overload (S6)

As it occurred in Stage 3, the organic overload of Stage 6 destabilized the culture, reducing biomass concentration, COD consumption, and PHA accumulation. On the other hand, glycogen accumulation and hydrogen production had the opposite tendency to achieve maximum H$_2$ productivity at 310 mLH$_2$ L^{-1} d^{-1}. The culture showed evident granulation, and the EPS accumulation increased to more than 10% dry weight. PPB abundance was again reduced to less than 20%, confirming that an organic overload destabilizes the PPB-enriched culture. The trends observed in Stage 3 are

repeated in Stage 6, confirming the reproducibility of the results and the culture's behavior under organic overload.

Figure III.7 Photos showing MPBR (top left), biomass collected at S2 (top right), lyophylized biomass (bottom left), and granulated biomass collected at P3 (bottom right).

The trends observed at each Stage depicted in Figure III.4 are also corroborated when we observe the performance parameters in Table III.1. The highest PHA productivities occurred at Stages 2 and 5, while higher glycogen and hydrogen productivities occurred at Stages 3 and 6, matching with the organic overload. Biomass yields in the best performing Stages (2 and 5) of 0.97 and 0.94 gCOD gCOD^{-1} were obtained, respectively. It is assumed that when the biomass yield drops, the non-consumed COD is being diverted to H$_2$ production. This fact agrees with the experimental data where we see the highest hydrogen yields and the lowest biomass yields at the same Stages. A maximum -q_s of 1 gCOD L^{-1} d^{-1} was achieved in Stage 5, but, interestingly, this parameter was not significantly reduced in the organic-overloaded Stages. As discussed previously, NH$_4^+$ is 100% consumed in Stages 1, 2, 4, and 5. Although it was not wholly consumed in Stages 3 and 6, its assimilation was still very high, up to 94 and 93%, respectively. However, PO$_4^+$ was not fully consumed in any Stage, with Stages 5 and 6 having the lowest consumption, down to 60%. It was

again confirmed that PHA is inversely related to glycogen accumulation and hydrogen production.

Table III.1 MBPR performance parameters on each Stage. Average ± standard deviation.

Parameters	S1	S2	S3	S4	S5	S6
$Y_{x/s}$ (gCOD gCOD^{-1})	0.83 ± 0.07	0.97 ± 0.05	0.62 ±0.08	0.86 ±0.25	0.94 ± 0.02	0.62 ± 0.07
$-q_S$ (mgCOD L^{-1} d^{-1})	541 ± 45	638 ± 32	677 ± 62	688 ± 28	1055 ± 66	665 ± 49
q_{PHA} (gPHA L^{-1} d^{-1})	0.22 ± 0.05	0.45 ± 0.05	0.05 ± 0.02	0.28 ± 0.02	0.61 ± 0.06	0.06 ± 0.04
q_{H2} (mlH$_2$ L^{-1} d^{-1})	88 ± 12	71 ± 15	140 ± 25	83 ± 26	51 ± 5	256 ± 34
q_{GLY} (gGLY L^{-1} d^{-1})	0.10 ± 0.03	0.09 ± 0.01	0.19 ± 0.04	0.09 ± 0.00	0.13 ± 0.01	0.17 ± 0.02
NH$_4^+$ comsuption (%)	100 ± 1	100 ± 1	94 ± 2	100 ± 2	100 ± 1	93 ± 1
PO$_4^+$ comsuption (%)	81 ± 9	85 ± 7	71 ± 3	84 ± 2	68 ± 7	60 ± 4

4.III.3. STATISTICAL ANALYSIS OF THE EVOLUTION OF MICROBIAL COMMUNITY PROFILES

Figure III.9 shows the development of the microbial communities evaluated by PCA ordination based on Hellinger transformed rarefied abundance. This analysis showed an apparent clustering of the samples according to their Stage and temporal progression. The major statistical difference occurred in Stages 1, 3, and 6. In Stage 1, the start-up with the synthetic feed caused a high enrichment in PPB. Inversely, in Stages 3 and 6, an organic overload produced the opposite effect. In terms of genera, we can observe a relationship between *Rhodopseudomonas* sp., *Rhodobacter* sp., and *Dsyngomonas* sp., which had more significant weight in the first three Stages. *Rhodophila* sp. and *Rhodovibrio* sp. had a greater weight in Stages 2 and 5, respectively. *Paludibacter sp.* and *Desulfovibrio sp.* were correlated and had the highest weight in the first organic overload (Stage 3). Also, they had a higher weight in the second one (Stage 6), which could mean cross-contamination *Sphingomonas* sp. and *Mesorhizobium* sp. which are some of the species present in the acidogenic fermenter (Figure III.8). which are less specialized in thermophilic temperatures and can therefore survive more efficiently in the MPBR. The clustering of the Stages and how most PPB genera were negatively related to organic overloads was confirmed. To further analyze these results,

an RDA was carried out to assess the contributions of environmental variables to variances in the microbial communities.
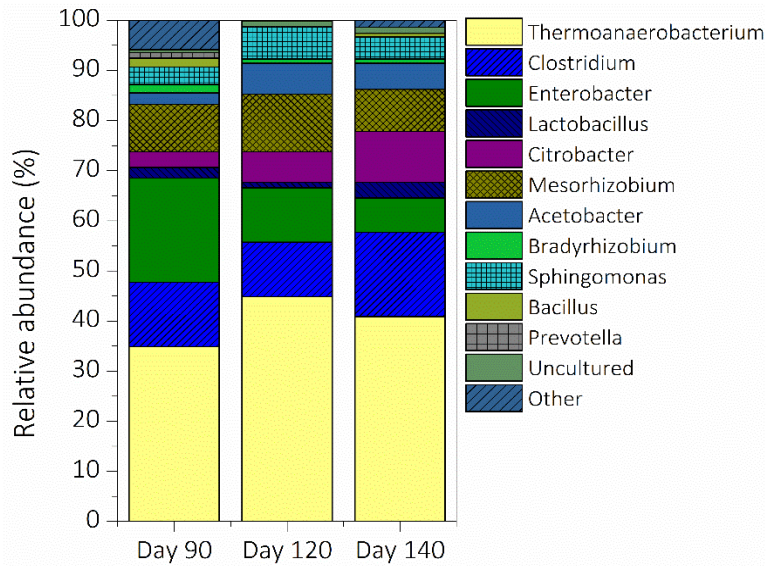
Figure III.8 Microbial community structure at genus level of the thermophilic fermentation reactor at 3 different days after stabilization.
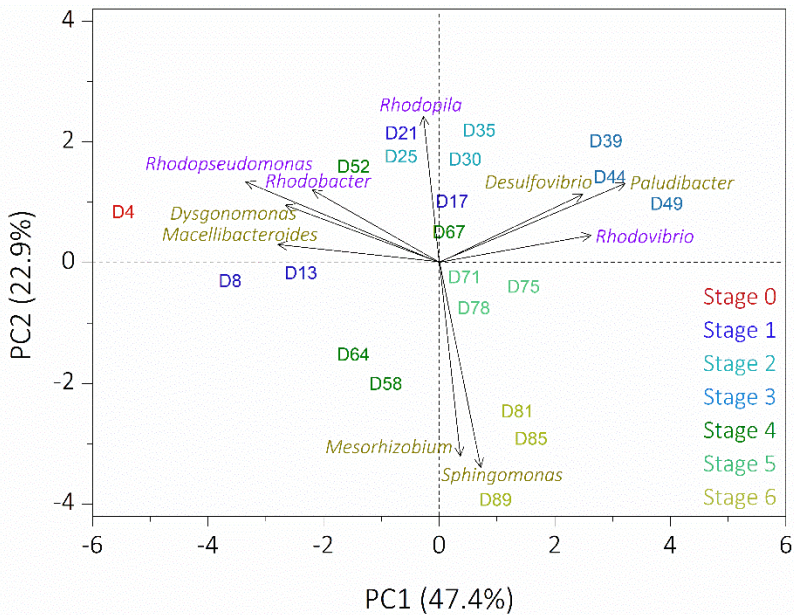
Figure III.9 PCA plot of the microbial communities. The samples are shown on day code (D4: day 4) and colored according to the Stage. Names indicate the 10 genera that contribute the most weight to PC1 and PC2, colored according to PPB (purple) or another genus (yellow). The arrows indicate the weight of each genus in each PC.

RDA analysis, shown in Figure III.10, confirms the relationship of PPB genera (*Rhodopseudomonas sp., Rhodobacter sp., Rhodopila sp., and Rubrivivivax sp*) with PHA accumulation and SRT and its inverse relationship with organic load (OLR). Also, *Acetobacter sp.* is highly correlated to PHA accumulation. Furthermore, the inverse relationship between PHA, glycogen, and hydrogen is again evident. A positive relationship is also observed between higher biomass concentration (VSS) and *Paludibacter sp.* and *Rhodovibrio sp.*
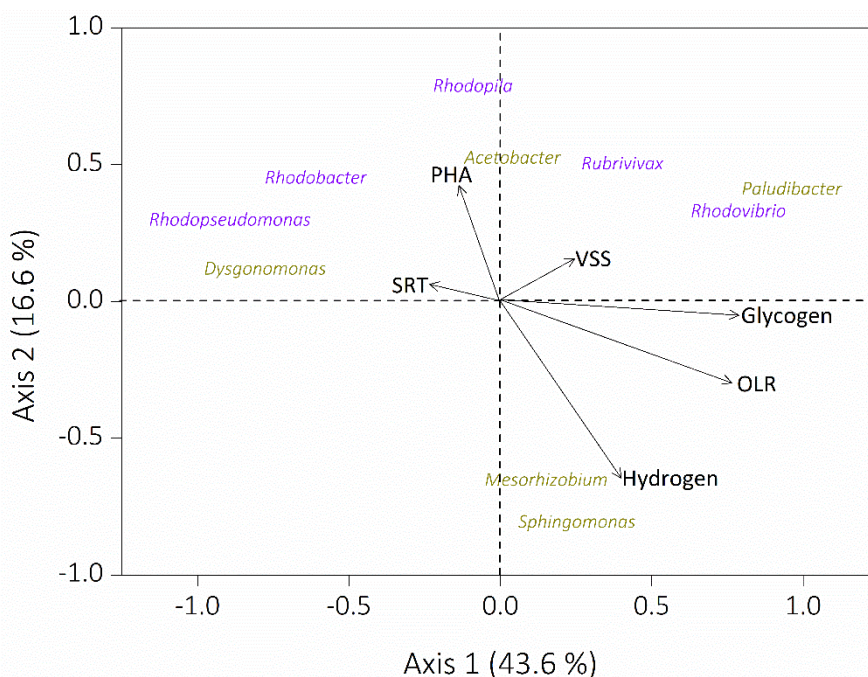
Figure III.10 RDA of bacteria related to environmental variables: PHA (gVSS$_{PHA}$ L^{-1}), SRT (d), VSS (gVSS L^{-1}), Glycogen (gVSS$_{GLY}$ L^{-1}) OLR (d) and H$_2$ (mLH$_2$ L^{-1}). Nodes show the 10 genera with the most weight in both axis (PPB in purple, other genera in yellow), and the arrows indicate the weight of each environmental variable.

To find more evident trends, another RDA analyzed only the statistical weight of time and OLR on the composition of the bacterial communities. The PPB *Rhodopseudomonas sp., Rhodobacter sp. and Rhodopila sp*., as well as *Acetobacter sp.* and *Dsyngomonas sp.*, are not affected by the time or OLR (Figure III.11). However, we can see that *Mesorhizobium sp.* and *Sphingomonas sp.* are positively related, indicating a possible development of contamination from the acidogenic fermenter developed over time. In a similar case, the PPB that are gaining ground with time are *Rubrivivax sp.* and *Rhodovibrio sp.*, but also *Paludibacter sp.,* not only associated with time but also with higher organic loads. Finally, fermentative genera had a great weight on hydrogen production.

Therefore, PHA and SRT are inversely related to hydrogen, glycogen, and OLR production, with different fermentative genera having the most weight for the latter environmental factor.
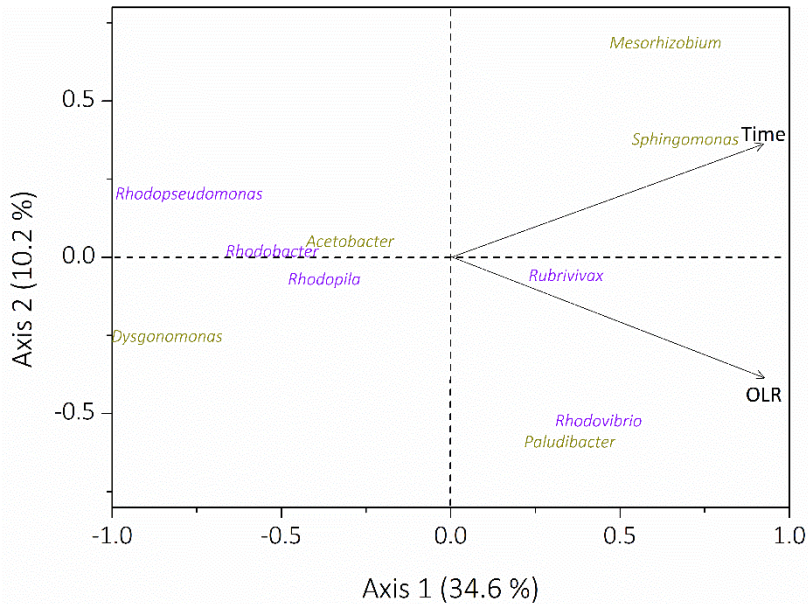
Figure III.11 RDA diagram of bacteria related to environmental variables: Time (d) and OLR (d). The nodes show the 10 genera with the most weight in both axis (PPB in purple, other genera in yellow), and the arrows indicate the weight of each environmental variable.

4.III.4. DISCUSSION

The results obtained in the combination of steam explosion and acidogenic fermentation pretreatments (0.66 $gCOD_{SCCA}$ gCODfeed^{-1}) can be considered a very efficient step in the PHA production pathway. This result is slightly lower than that obtained recently in acidogenic fermentation of fruit residue, where a yield of 0.74 $gCOD_{SCCA}$ $gCOD_{feed}^{-1}$ was achieved (Matos *et al.*, 2021). Another work showed fermentation of OFMSW to obtain an overall yield of 0.65 ± 0.04 $gCOD_{VFA}$ $gVS_{(0)}^{-1}$ and acidification of 86% (Moretto *et al.*, 2020). In our work, we obtained slightly higher acidification (90%), which we know from previous work is essential when working with PPB since the high presence of sugars in the substrate can negatively affect the accumulation of PHA (Almeida *et al.*, 2021). The remainder of the undefined COD could be oligo- and monosaccharides derived from hemicellulose, lipids, proteins, complex carbohydrates, and amino acids (Romero-Cedillo *et al.*, 2017). Within the SCCA produced, acetic acid corresponded to 43.4% of the COD-equivalent. Previous studies

determined that it is the most suitable substrate for PHA production with PPB (Fradinho *et al.*, 2014). The second SSCA was butyric acid, corresponding to 25% of the COD-equivalent of SCCA. Propionic, valeric, and hexanoic acids represented 17, 7, and 7%, respectively. It is well-known that the mixing of acids favors the production of copolymers; for example, propionic and valeric acid favors the accumulation of PHV (Fradinho *et al.*, 2019), and although it has not yet been demonstrated in a PPB mixed culture before, it has recently been discovered that the presence of hexanoic acid promotes the accumulation of PHH in *Rhodospirillum rubrum* (Fradinho *et al.*, 2019). If these results are kept in the process scale-up, the SCCA productivity and the fast reaching of a steady-state, reproducibility, and process stability highlight the practicality and high suitability of these pretreatments for PHA production with PPB.

The PPB-enriched mixed culture accumulated high percentages of PHA by assimilating fermented effluent from OFMSW feed. The maximum percentage of PHA accumulation achieved was 42%, which is the maximum achieved with mixed cultures of PPB using residual substrates (Sali and Mackey, 2021), the closest result being the treatment of fermented domestic wastewater, where 30.8% was achieved (Almeida *et al.*, 2021). Figure III.9 shows an unequivocal relationship between the abundance of PPB genera and PHA production. *Rhodopseudomonas* sp. is the most abundant PPB genus in all Stages except S3, reaching up to 90% of the total, and coincides with the most abundant genus in other studies performed in our laboratory (de las Heras *et al.*, 2020). In other studies, the bacterial communities fluctuate in their percentage of PPB, for example, from the dominance of *Rhodopseudomonas* to *Rhodobacter* (Hülsen *et al.*, 2016), and although *Rhodobacter* sp. is the second most abundant PPB genus identified, in our study, *Rhodopseudomonas* sp. always remained predominant among PPB. *Rubrivivax* sp. is the PPB genus with the highest reported PHA accumulation, with 85% by weight in pure cultures (Sali and Mackey, 2021), which coincident with the high PHA accumulations in Stage 5. In addition, we can see a recurrent closeness between these PPB and the genera of *Dsyngomonas* sp. and *Acetobacter* sp., which may indicate symbiotic processes. *Acetobacter* sp., for example, can oxidize some short-chain acids such as lactate and butiryc acid and ethanol to acetic acid (Nakano and Fukaya, 2008), and PPB can assimilate this substrate to accumulate PHA faster. A more comprehensive metaproteomic study could help to clarify these symbiotic processes in enriched mixed cultures since it is essential to know which PPB species have the highest capacity to accumulate PHA to improve this process.

Among the different PHA monomers produced, PHB is the predominant one, but other less common ones, such as PHH, reach relevant percentages.

Most PHA accumulation studies with PPB only considered PHB and PHV accumulation. In our work, we found, in addition to PHB and PHV, the PHH in all Stages except Stage 0. The rest of the Stages accumulated between 2% and 30% of the total PHA in dry mass. It was demonstrated that PHH is accumulated when hexanoic acid is present in the medium, assimilated via β-oxidation, and incorporated into PHA chains (Silva *et al.*, 2022). In addition, the presence of hexanoic acid increases the accumulation of PHB by boosting the production of intermediates such as Acetyl-CoA and butyryl -CoA (Cabecas Segura *et al.*, 2022). In Stage 0, PHH was not detected because the synthetic substrate did not contain hexanoic acid. In the rest of the Stages, PHH had similar behavior to the rest of the PHA. Only two papers reported PHH production by phototrophic bacteria, one where C6 and C7 monomers were detected in *Rhodospirillum rubrum* (Brandl *et al.*, 1989), and another where PHH was detected in a mixed PPB culture (Liebergesell *et al.*, 1993). In addition to the PHH, we detected another peak in the GC, and, considering its retention time, we assumed that it could be a 3-carbon monomer forming polyhydroxypropionate (PHP). This assumption was furtherly verified on GC-MS, and Figure III.12 depicts the results. Both PHH and PHP, which are generally not accounted for, indicate that PHA accumulation is usually underestimated with mixed PPB cultures, which further increases the interest in the technology.
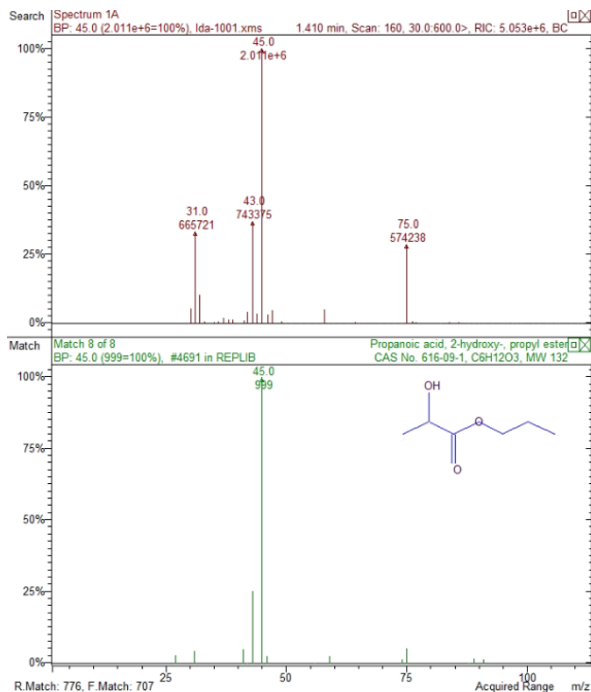
Figure III.12 GC/MS mass spectra and fractionation pattern. The pattern above is the analyzed sample, and the pattern below is the theoretical polyhydroxypropionate compound in its esterified form.

One of the significant limitations of advancing this technology is the low productivity obtained so far. In this study, PHA productivity reached very high values (q_{PHA} up to 0.61 gPHA L^{-1} d^{-1}) compared to previous works. The highest data obtained to date in mixed cultures of PPB is 0.77 gPHA L^{-1} d^{-1} (Fradinho *et al.*, 2019), but being fed with acetic acid. In a recent study by the same group using fermented wastewater, the highest productivity obtained with a permanent feeding regime was 0.23 gPHA L^{-1} d^{-1} (Almeida *et al.*, 2021). Although the results presented in this work are promising, they are still far from the productivities obtained with aerobic cultures, as they report average productivities of 5-10 gPHA L^{-1} d^{-1} (Capson-tojo *et al.*, 2020). The primary constraint is biomass growth, which is limited by nutrients in this work. However, the higher the biomass concentration, the more this technology is limited by the volumetric irradiance in the reactor. In this work, a submerged LED lamp with a very low volumetric irradiance (2.1 W L^{-1} or 5 W m^{-2}) was used, when most studies are usually above 30 w m^{-2} (Fradinho *et al.*, 2021). Using the biomass productivity of Stage 5 (1.8 gVSS L^{-1} d^{-1}), this would result in a biomass energy yield of 61 gCOD kWh^{-1}, which is slightly higher than the highest

recorded so far of 59 gCOD kWh^{-1} (Capson-tojo *et al.*, 2020). New lighting systems and reactor configurations (especially systems to retain solids) should be investigated to produce more concentrated biomass. Another critical element is increasing PHA accumulation and understanding what other components, carbon, electrons, or both, can be diverted when PHA accumulation ceases.

The increase of organic load in the MPBR led to the destabilization of the culture and the collapse of PHA accumulation (Stages 3 and 6, Figure III.4). An increase in glycogen accumulation and hydrogen production was detected in both Stages. This evidences the competition between carbon allocation to growth and accumulation of PHA and glycogen, with electrons allocation between growth, PHA accumulation, and hydrogen production (Figure III.13). Glycogen accumulation by PPB has been known for a long time. Still, only one study has examined the relationship between PHB and glycogen production (Philippis *et al.*, 1992), showing that PHB is an electron sink that works as an intracellular reserve for reducing power. However, it rapidly decreases when biomass growth is reduced, thus diverting carbon storage to glycogen accumulation.
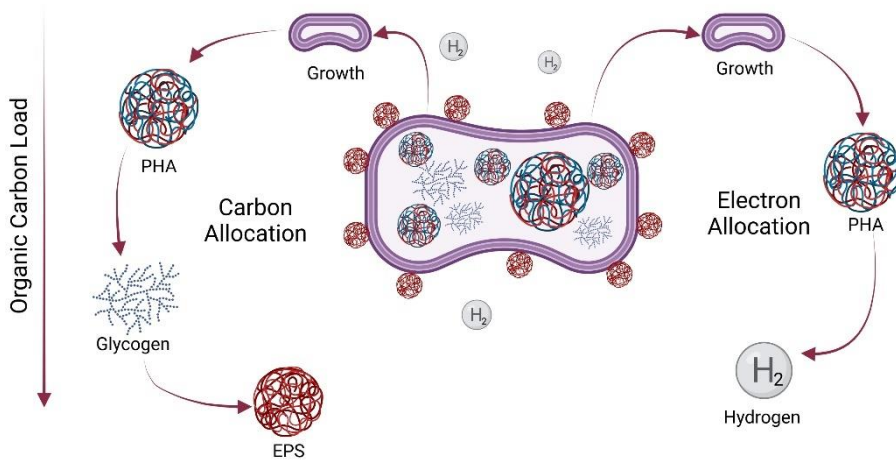

Figure III.13 Schematic description of the different biopolymers for carbon storage and consequent electron allocation between PHA, glycogen, EPS, and hydrogen, related to increasing organic load stress.

A variety of metabolic principles are linked to glycogen metabolism: (i) the maintenance of photosynthetic efficiency in light and (ii) of viability in periods

of starvation, such as in darkness or macronutrient depletion, and (iii) the acclimation to macronutrients deficiency (Mas and Van Gemerden, 1995). It is noteworthy that after Stage 3 and the lowering of OLR, there was a rapid recovery of the culture, demonstrating that the PHA production is stable and resistant to severe organic stress, and although the community within the PPB mixed culture may change, its functionality remains consistent. However, once PHA is no longer available as an electron sink, hydrogen production appears as a possibility for the balance of reducing equivalents.

The competition between hydrogen production and PHA accumulation in *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* strains has been observed (Hustede *et al.*, 1993). The conversion of PHB accumulated in *Rhodovalum sulfidophilum* to hydrogen in the absence of other substrates was demonstrated (Maeda *et al.*, 1997). In our experiment, this inversely proportional relationship is evident in Figure III.9. However, the mechanisms of electron transfer and how both metabolic pathways compete for electrons allocation are poorly understood. While the decreasing trend in PHA and increase in glycogen accumulation and H_2 production is observed at Stages 3 and 6, biomass granulation was also evident, whereby at Stages 4, 5, and 6, the accumulation of EPS was measured. During organic overload of these stages, PPB lower their cellular efficiency and divert excess carbon and electrons to EPS and H_2, respectively. Another hypothesis for the increased hydrogen production is that EPS, which are exopolymers composed mainly of carbohydrates, may be optimal substrates for the growth of fermentative bacteria. These fermentatives can come from cross-contamination between bioreactors as explained in previous sections, increasing their biomass over time and producing more H_2. In addition, the COD of the biomass decreases considerably, as well as the cell yield, which is linked to fermentative processes. Fermentative bacteria produce more oxidized compounds for PPB, which can assimilate them for growth, causing a symbiotic relationship already evidenced in Figures III.9 and III.10. The strategy of achieving a granular culture of PPB first and then deriving its metabolism to PHA production could lead to considerable cost savings downstream of the process, as granular sludge can decrease the Operational Expenditure (OPEX) of the process by up to 50%, which has been reported for other anaerobic bacteria (Tavares Ferreira *et al.*, 2021).

The granulation capacity of PPB has been studied recently, and EPS accumulations of up to 35% in dry mass were observed (Stegman *et al.*, 2021). At Stage 6, a 10% dry mass of EPS was reached. EPS are primarily composed of

carbohydrates, proteins, and lesser amounts of other components and have multiple roles such as flotation and locomotion, feeding, protection against desiccation/UV/pollution, development of biofilms and communication, and are widely being employed at industrial scale in cosmetic, pharmaceutical and petroleum industries (Sreejita Ghosh *et al.*, 2021). In the work where PPB granulation is analyzed, the relative abundance obtained is between 40 to 70% (Stegman *et al.*, 2021), slightly higher than obtained in this work, where the range moves from 22 to 80%. The relationship between PHA and EPS in PPB has not been studied so far, but the negative correlation between EPS and PHA was evidenced in other microorganisms (Zhao *et al.*, 2021), as shown in Figure III.4 of our study. However, it is known that the EPS confer protective effects upon the cell, allowing microorganisms to grow in attached mode and improving the settling. Although a study showed that the settling cohesion was enhanced and stabilized by PPB (Larson *et al.*, 2009), little is known about the stabilization and sedimentation in PPB cultures, even less with high EPS content. Considering that the downstream processing, including settling and biomass extraction, is a bottleneck in PHA production (Fernández-Dacosta *et al.*, 2015), **the granulation of mixed PPB cultures and the simultaneous production of PHA and EPS can reduce costs in the scale-up of the technology** (Kopperi *et al.*, 2021) **and opens up exciting research opportunities for the future.**

4.III.5. Implications for an integrated photo-biorefinery

Cascading biorefineries for waste treatment are indispensable for a sustainable future because they can turn a problem into an opportunity. The innovative biorefinery platform proposed integrates a steam explosion pretreatment that can significantly increase the fraction of fermentable organic carbon from the OFMSW with an acidogenic fermentation that produces high yields of SCCA, which are the perfect substrate for PHA accumulation in a PPB-enriched culture. This biorefinery is completed with the anaerobic digestion of the solid fraction obtained after fermentation, yielding 336 LCH_4 $kgVS^{-1}$ (Figure III.14). This additional energy source would close the carbon and energy cycle of the biorefinery.
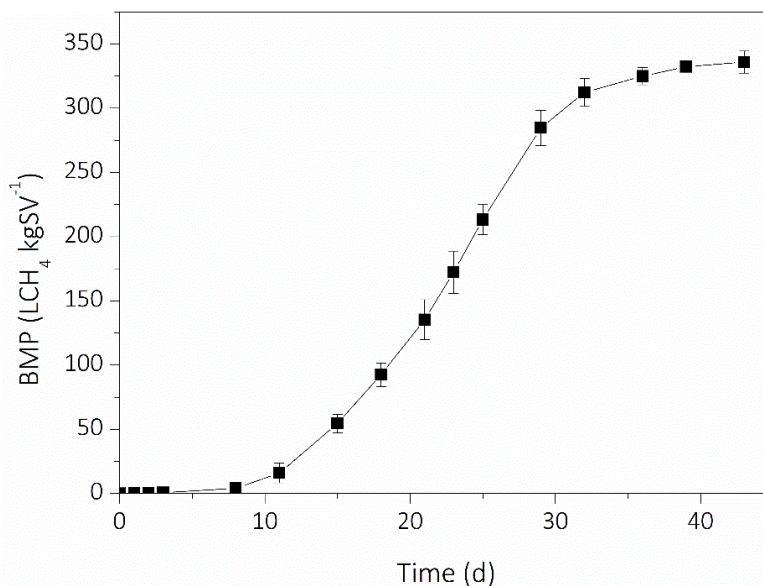
Figure III.14 Biochemical potential test (BMP) of the solid phase obtained after the acidogenic fermentation pretreatment. Error bars are 95% confidence intervals.

As for the COD removed, on average more than 40% of the COD is not assimilated, mainly due to nutrients limitation. This residual COD flow from the MPBR could be combined with the final digestate from anaerobic digestion, a high nutrient side stream, in a possible second phototrophic reactor with PPB dedicated to protein production. This technology has already been tested several times (Alloul *et al.*, 2019; Delamare-Deboutteville *et al.*, 2019). Nonetheless, the biggest challenge of the technology shown in this Thesis is the industrial scalability of the MBPR.

4.III.6. MASS AND ENERGY BALANCE OF A CONCEPTUAL PHOTOBIOREFINERY

The industrial production of PHA is currently carried out by pure aerobic cultures based on sugars or other similar substrates. Comparatively, the production of PHA with mixed PPB cultures has some advantages: (i) no need for sterilization of equipment, (ii) they can use a wide variety of waste as substrate, eliminating a large part of the production costs, (iii) they do not need aeration, (iv) higher yields of PHA are possible, and (v) they can accumulate and produce PHA in the same reactor by IR illumination, eliminating the requirement for sorting and an accumulation reactor. An intermediate

approach is aerobic mixed cultures, which are already being studied at a pilot plant scale (Matos *et al.*, 2021; Moretto, Lorini, *et al.*, 2020). This technology's best performance achieved so far is 0.45 gCOD$_{PHA}$ gCOD$_{Feed}^{-1}$ (Matos *et al.*, 2021). A conceptual photobiorefinery has been proposed (Figure III.15). Based on the data obtained in this section a preliminary mass balance of the overall novel photobiorefinery proposed here shows a total yield of **109 kgPHA tonneTS^{-1} (0.15 gCOD$_{PHA}$ gCOD$_{Feed}$)**, which is two orders of magnitude above that achieved in the proof of concept in Section I. However, it is 3 times less than the aerobic process, but anyway we have to take into account that it is a far more mature technology that has been studied for several years. Moreover, although this is the highest biomass energy yield seen so far, as discussed in previous sections, for the time being, the production of PHA with PPB and artificial illumination is considered to be economically unfeasible (Capson-tojo *et al.*, 2020), the most logical solution being the scaling of the technology using the irradiance of the sun. Another important consideration of this balance is that we obtain a combined production between the acidogenic and photoheterotrophic fermentation process of **347 kg H$_2$ tonneTS^{-1}** treated. However, scaling up outdoors involves many uncertainties, such as contamination by other microorganisms, response to non-fully anaerobic systems, and intermittent solar illumination. **These problems could be solved by sunlight collection and filtration systems, as discussed in Section IV. Operational strategies adaptable to the different seasons of the year and the feedstock arriving in the biorefinery is a crucial point for its development.**

Several streams should be accounted for and recirculated/recycled within the proposed integrated photobiorefinery, as illustrated in Figure III.8. For example, the photoheterotrophic process requires an 11-fold dilution, thus requiring a significant amount of water. The aqueous stream remaining after the PHA extraction process could be used to save water and costs. Also, the membrane output stream has a high organic content, basically composed of SCCA. This stream would have several potential applications, such as the purification of the SCCA or their use in another photoheterotrophic PPB reactor in combination with a nutrient source for the production of microbial protein, for instance. Furthermore, in the interest of completely closing the carbon cycle, we have proposed that the share of biomass (TS) remaining after PHA extraction can be can be recirculated to the anaerobic digestion process, increasing biomass production. To calculate the methanogenic potential we

take as reference the value of 210 mLCH$_4$ gVS^{-1} determined by Hülsen *et al*. (2020).

If we only estimate the anaerobic digestion process based on the ST from the acidogenic fermentation, another major point to note is the reduction of the solid residue by up **to 90%, with the production of 91 kg of digestate per tonneTS**. Nonetheless, since we add the biomass remaining from the PHA extraction, the digestate increases up to 141 kgTS, but would also increase the nutrient content of this digestate, improving its potential applicability as an organic fertilizer.In any case, this digestate could be used as an organic soil amendment, or even the soluble fraction could be recycled together with the high-organic content stream in order to produce proteins, as mentioned above.

We can conclude from the preliminary balances that the anaerobic digestion and steam explosion pretreatment would be energetically autarkic. If we use the biogas produced in the anerobic digestion as proposed by Cano *et al.* (2014) and as described in Chaper 3, we would produce hot water with enough thermal energy to heat the anerobic digester. We would also produce up to **457 kWh of electricity per TonneTS**, which can be used for plant self-consumption or sold to the grid. And finally, the exhausted gases would be used to generate steam. This steam produced would be sufficient to provide the steam needed for the steam explosion pretreatment, leaving up to **234 kgSteam TonneTS^{-1}**.
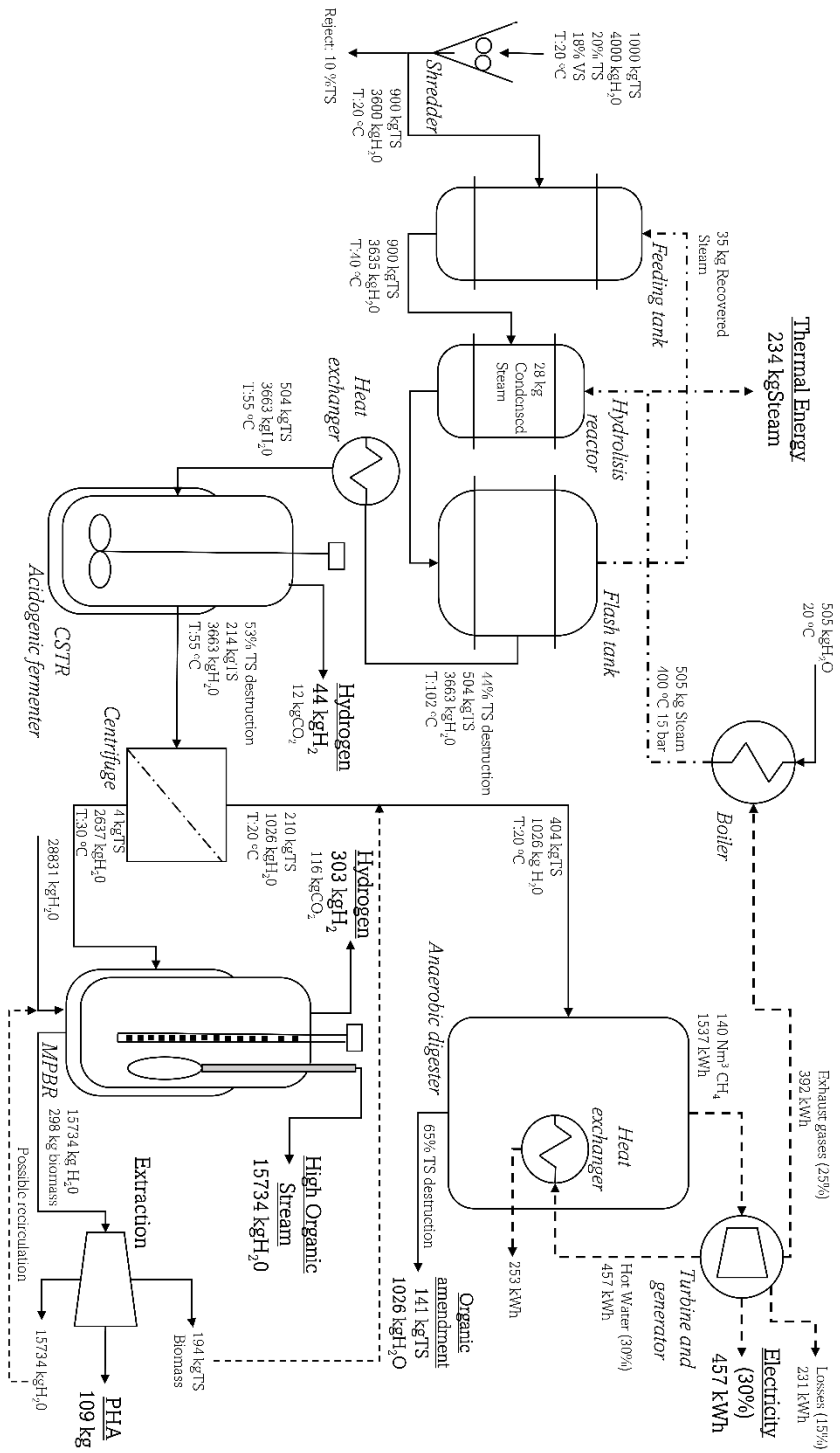
Figure III.15 Preliminary mass and energy balance of the proposed photobiorefinery

The possibility of producing different products seasonally (H_2, glycogen, EPS), in addition to PHA, can provide the system with more economic resilience. This study demonstrates that if the reactor is operated at a higher organic load, the carbon accumulation is redirected to glycogen, or even granulation can occur in the culture due to stress and derived to EPS production. This links to higher H_2 productivities through electron allocation in PPB metabolism or synergies generated with fermentative bacteria. In addition, this work opens the way for the production of longer chain biopolymers that could diversify their industrial applications, considering that the PHB-PHV-PHH polymers present rubber-like elastomeric properties and can therefore be used in a different set of applications than a polymer composed solely of PHB and PHV (Pereira *et al.*, 2019). Thus, it would still be essential for the scale-up of this technology to optimize PHA production and to be able to tune the type of polymer obtained. Finally, we evidenced which PPB genera are more critical in the PHA accumulation process and how although the community evolves, its functionality remains constant. All this Information gives us tools for optimizing and controlling this technology and paves the way for future large-scale feasibility analyses.

4.III.7. CONCLUSIONS

This study demonstrates the possibility of increasing PHA production by combining different operating strategies. The association of steam explosion pretreatment and acidogenic fermentation leads to stable production of SCCA and H_2 from OFMSW. Understanding the different metabolic pathways of carbon assimilation and consequent electron allocation is critical to enhancing PHA production. This production is shown to be stable and resistant to severe organic stress, and although the community within the PPB mixed culture may change, its functionality remains consistent. Finally, this comprehensive overview helps design a PPB-based pho-biorefinery with seasonal PHA, EPS, and H_2 production, depending on the culture feed, contributing to urban solid waste management and taking a step forward in the direction of a circular economy society.

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SECTION IV: SCALING-UP CHALLENGES OF PHOTOHETEROTROPHIC PROCESS

Preamble and Rationale

As discussed in the previous section, the scale-up of the photoheterotrophic process is key to achieving a cost-effective process. The successful scale-up for biopolymer production on an industrial scale depends on several factors, such as the cost of precursor substrates, yield over substrate rate, volumetric productivity, and the cost of downstream processing, among others. While bioengineering aims to improve upstream processes (low-cost substrates and increased productivity), bioprocess optimization of upstream and downstream processes is necessary for scalable and cost-effective manufacturing. In this last section of the discussion Chapter, we have considered reviewing and performing a critical analysis of the current state of the art in scaling up these PPB-based biopolymer production technologies.

4.IV.1. PPB STORAGE COMPOUNDS

Storage compounds are produced by living organisms and/or synthesized by processive enzymes that link building blocks to yield high molecular weight molecules. Figure IV.1 shows the main environmental conditions for the accumulation of storage compounds and their industrial applications are schematically shown in Figure IV.1. Depending on the conditions in which PPB are growing, five major storage compounds are produced: zero-valence sulfur, glycogen, PHA, and polyphosphate (poly-P). These compounds are stored intracellularly (and therefore are inclusions) except for sulfur and EPS, which can also be stored extracellularly.
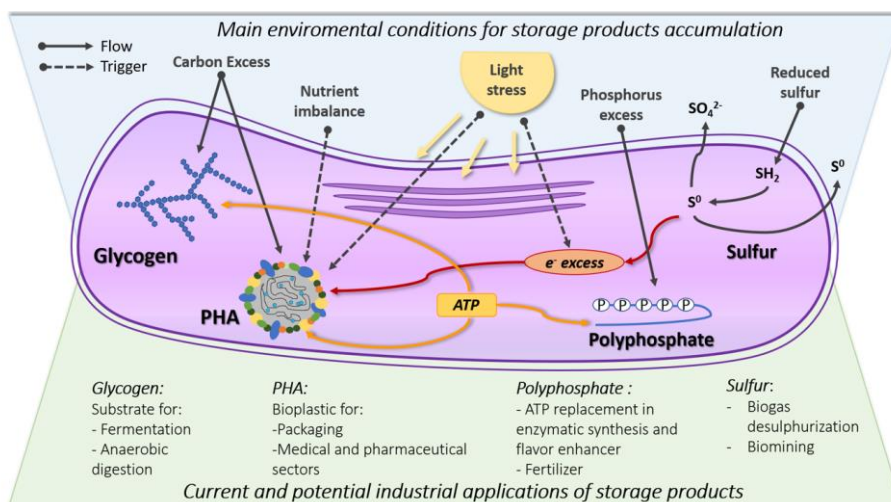
Figure IV.1 Schematic representation of PPB anaerobic phototrophic metabolism that support storage products accumulation and their industrial applications.

Poly-phosphate

Inorganic poly-P is the only polyanhydride found in all living cells forming linear polymers with variable chain length, which are constructed from repeating PO_4^{-3} and connected by high-energy anhydride bonds (Liang *et al.*, 2010). It has been shown that poly-P affects numerous aspects of bacterial physiology, such as survival during the stationary growth phase, response to stress, motility, quorum sensing, biofilm formation or pathogenicity. The enzyme responsible for poly-P biosynthesis is the highly conserved poly-P kinase (PPK), which helps to store energy excess from light through phototrophic growth when low C / P ratios are present (Lai *et al.*, 2017). Poly-P

forms intracellular storage particles but may also form a membrane-anchored complex with low molecular weight polyhydroxybutyrate (PHB), facilitating the uptake of DNA and various ions (Reusch and Sadoff, 1988). This polymer can be accumulated as P reserve in response to periods of P starvation, but some organisms can accumulate Poly-P as energy storage (ATP), without requiring a previous P starvation phase in environments with excess P. This capacity has been observed in phototrophs like microalgae (Solovchenko *et al.*, 2016), PPB (Lai *et al.*, 2017, Liang *et al.*, 2010) and heterotrophs like the poly-P accumulating organisms (PAOs) (Desmidt *et al.*, 2015). Some PPB species can accumulate internal PO_4^{-3} into the form of poly-P up to 13%–15% of its cell dry weight (Liang *et al.*, 2010), nevertheless, there is still a great gap of knowledge regarding its accumulation patterns on PPB. Unlike poly-P accumulating chemoheterotrophic organisms, the driver for poly-P accumulation in PPB is related to the presence of high light availability, similarly to other photosynthetic microorganisms (Carvalho *et al.*, 2019). Therefore, the process design is simplified as there is no need to have a succession of aerobic/anoxic stages in the photobioreactors, considerably decreasing the operation costs.

Phosphorus presents vast applications in the chemistry and food industry, but particularly, in the agriculture field where P is used as fertilizer (Solovchenko *et al.*, 2016). Commercial production of bacterial poly-P for industrial applications is not currently economically feasible due to a much more efficient production by chemical synthesis (Iliescu *et al.*, 2006). However, controlling the phosphorus propagation in nature is a highly important matter in environmental biotechnology, and the biological capture and recovery of P in waste is key for the protection of the aquatic environment. PPB systems are still under study for P recovery, which can boost the use of PPB biomass as C/N/P organic fertilizers (Sakarika *et al.*, 2020).

Glycogen

Glycogen, a polysaccharide made up by glucose units, is another intracellular stored resource common in evolutionarily divergent species. It is a widespread form of carbon and energy storage that promotes survival during starvation (Sekar *et al.*, 2020). Its role in bacteria is highly diverse, for example, it contributes as an energy source when there is a lack of other energy sources, or as a temporary resource used during the physiological transitions required by dynamic environmental conditions. It can be accumulated in either stationary phase or under excess carbon and/or limited-growth conditions, and

contributes to survival or maintenance in bacterial environments in which nutrient availability frequently fluctuates (Sekar *et al.*, 2020). The glycogen biosynthesis capacity of PPB has been already measured (Igarashi and Meyer, 2000). However, there is still a gap of knowledge in the relation between PHA, and glycogen accumulation in PPB, as addressed in this Thesis. A previous study indicated that *Rhodopseudomonas palustris* accumulate glycogen in higher percentage in almost all conditions (5–15% d.w.), synthesizing PHA when the environment has reducing equivalents in excess (0.3–7% d.w.) and the PPB are in static mode (De Philippis *et al.*, 1992). However, glycogen accumulation remains almost constant, with decreasing values related to starvation conditions. The possible competition between glycogen and PHA accumulation in photoheterotrophic conditions, as well as the genetic regulation for this process, is still unclear. We may hypothesize that glycogen acts always as a carbon and energy storage during growth, whereas PHA regulates the electron balance, acting also as carbon storage during static mode. The energy required for glycogen is higher than that for PHB accumulation (starting from acetate, 1 mol of glycogen requires 4 ATP, 2 CO_2 and 4 reducing equivalents, whereas 1 mol PHB only requires 2 ATP and 1 reducing equivalent (Fradinho *et al.*, 2014)). However, glycogen is on the polysaccharides biosynthesis pathway, and therefore it is the natural carbon storage process during growing conditions. PHA accumulation is more feasible in static mode, requiring less energy. Also, the lack of CO_2 is a trigger for PHA accumulation in detriment of glycogen, as Acetyl-CoA needs CO_2 for initializing any biosynthetic pathway (Bayon-Vicente *et al.*, 2020).

The use of PPB-based glycogen as a carbon source for a variety of applications remains untapped. Recently, it has been studied the accumulation of glycogen in cyanobacteria to use the bacterial biomass as a feedstock for octanoic acid through dark fermentation (Comer *et al.*, 2020), which may be a feasible alternative for PPB. In addition, the anaerobic digestion of PPB-based biomass has been recently studied (Hülsen *et al.*, 2020). The role of glycogen in this process should be stated as it can potentially boost the biochemical methane potential of PPB biomass rejection coming from the extraction of higher value-added products. In this sense, glycogen may be a byproduct of biorefinery platforms. In any case, it interferes with most of the metabolic pathways of PPB and must be considered in any biopolymers' application of PPB.

Sulfur

PPB have the ability to grow using reduced sulfur compounds (mainly H_2S) as electron donors for their biosynthesis (Pokorna and Zabranska, 2015). Sulfide is oxidized to sulfate and, as an intermediate, elemental S_0 is produced and accumulated in the form of globules inside or outside the cells. In principle, S_0 can serve as both an electron acceptor (when it is being accumulated) and as a donor (Trüper, 1984), therefore sulfur compounds, and S_0 in particular, is expected to have a considerable influence on the production and/or consumption of H_2 by PPB (Laurinavichene *et al.*, 2007). An excess of sulfur in the environment can induce sulfur accumulation of over 30% of the dry weight of the cell (Pedrós-Alió *et al.*, 1985).

Although there is no industrial scale of sulfur removal by PPB so far, this is a promising technology that can be used, for example, for biogas desulphurization. A previous study performed in the laboratory and in a pilot plant, achieved a complete oxidation of H_2S of the biogas using the purple sulfur bacterium Ectofhiorhodospira shaposhnikovii (Vainshtein *et al.*, 1994). A recent paper have shown potential capacity of mixed cultures of PPB for photoautotrophic sulfide removal where the purple sulfur bacterium *Allochromatium* sp. predominated in the consortium (Egger *et al.*, 2020). In this sense, mixed cultures of PPB may allow a potential technology for simultaneous wastewater treatment and biogas upgrading, including both biogas desulphurization, removal of carbon dioxide, and organic matter and nutrients removal (Marín *et al.*, 2019).

Biomining is another option to use the ability of PPB to transform sulfide into sulfur globules. These organisms may be useful as intermediates in bio leaching of metals after metal recovery by sulfate-reducing microorganisms in a two-stage biological approach. Though this has been never tested with PPB, there are evidences indicating that sulfide-oxidizing microorganisms are suitable for these purposes (Suzuki, 2001).

Polyhydroxyalkanoates (PHA)

This Ph.D. Thesis is mainly focused on the production of PHA, which are linear polyesters, formed by the accumulation of carbon as reserve material in response to substrate excess when growth is limited owing to starvation of some nutrient, usually nitrogen, phosphorus or sulfur (Monroy and Buitron, 2020). PHA is deposited as spherical intracellular inclusion with an amorphous

and hydrophobic PHA core that is mainly surrounded by proteins involved in such PHA metabolism (Jendrossek, 2009). PHA accumulation occurs in high-carbon and nutrient-limited (N, P, S) environments, but it is also enhanced in case of redox imbalance, to act as an electron sink (Bayon-Vicente *et al.*, 2020, De Philippis *et al.*, 1992), therefore sudden light increase could be a way to induce redox stress in the culture, thus forcing higher PHA accumulation. However, long-term cultivation could lead to metabolic adaptation and eventual decrease of PHA accumulation.

The most investigated PHA is the homopolymer poly-3-hydroxybutyrate (P3HB). Compared to the P3HB, PHA copolymers, composed of a mixture of hydroxybutyrate and hydroxyvalerate (PHBV), show better mechanical properties with decreased stiffness and brittleness, increased flexibility and a decreased melting and glass transition temperatures, which allows for a wider temperature processing window and thus increase its processability (Albuquerque *et al.*, 2011). A thorough review of PHB (homo- and copolymers) production with PPB mixed cultures has been recently published (Monroy and Buitron, 2020), where it has been shown the copolymer production capacity of PPB when using heterogeneous substrates (propionate among others), with up to 51% fraction of PHV moiety (Fradinho *et al.*, 2014).

As discussed in Chapter 1, among bioplastics, PHA has the best biodegradability even in marine environments, but this feature has been barely considered when the production costs are calculated in cradle-to-grave approaches (e.g. life cycle assessment). An important aspect to consider in this context is the production by PPB of hydrogen as a by-product in the accumulation of PHA. This process have been widely studied in different bioreactors (Basak *et al.*, 2014), and can be a possible strategy to improve the profitability of the overall process as has also been demonstrated in Section III. A solar-powered photo-biorefinery could be as well a promising way for sustainable biodegradable PHA production, as will be discussed in subsequent sections.

4.IV.2. ENRICHING PHOTOTROPHIC MIXED CULTURES IN PHA ACCUMULATING PPB

In order to develop strategies that promote PHA production in PPB mixed cultures, it is essential to recall that in PPB, PHA can be stored as a carbon

reserve and as a sink for reducing power. Therefore, strategies can be designed by specifically targeting each function. Up to now, studies have been mainly conducted with synthetic feedstock and indoor artificial illumination, creating a knowledge base for higher complex system operation with real wastes in outdoor conditions.

The two most important strategies used for the production of PHA are feast-famine and permanent feast and their key features are summarized as follows:

The **feast and famine strategy (FF)** is widely identified as an prominent method for PHA accumulation by mixed microbial culture within aerobic systems. This processes are usually based on the alternance of periods in which substrate (energy/C source) is available, named feast phase, and periods in which substrate is no longer available, named famine phase. The aim of these processes is pushing towards the survival of the of the most resistant strains (strains that under this cultivation condition accumulate intracellular energy and reserve compounds like PHA in the feast phase, and then use them as energy substrate in the famine phase) in place of the survival of the faster growing strains (Montiel-Corona and Buitrón, 2021), otherwise obtained with culture medium fully replete in every nutrient. This type of strategy is typically used in SBR reactors or two continuous CSTR reactors (Di Caprio, 2021). There are two main ways of operation:

1)   Feast and famine with coupled C/energy and N supply: In this strategy the organic substrate (C and energy source) is supplied together to the N substrate, at the beginning of the cultivation (feast phase). The C/energy source is quickly consumed. When the C/energy source is depleted, cells enter in the famine phase, in which they can survive depending on its duration and on the amount of the compounds previously storage.

2)   Feast-famine with uncoupled C/energy and N supply: In this strategy there is a feast phase corresponding to the supply of C/energy source in N-starvation, followed by a famine phase corresponding to the supply of N in absence of the external energy source. In the feast phase, in N-starvation, the cell duplication is arrested, and the biomass concentration increases because of the sole accumulation of organic compounds. For phototrophic cultivation of PPB this uncoupling could be attained easier because the energy source used is light, that was just turned off at night.

The other main strategy would be to maintaining the culture in a carbon feast regime, with permanent presence of external carbon. The selection principle of this **permanent feast** (PF) relies on the distinctive properties of

anoxygenic photosynthetic bacteria, where no oxygen is released during photosynthesis. In illuminated environments, photosynthetic bacteria can use the ATP produced by photosynthesis to uptake external carbon. If no electron acceptors are present, the cells have to activate internal mechanisms to oxidise the reduced molecules. One of them is the accumulation of PHA that requires the reduction of its precursors during polymer formation. Thus, PPB are able to grow exponentially and accumulate PHA simultaneously (Montiel-Corona and Buitrón, 2021). In addition, the selection and accumulation process can be carried out in a single reactor, since in this reactor would be simultaneously a selector and a PHA accumulator reactor, where organisms would be permanently selected while accumulating PHA, which can potentially reduce production costs (Fradinho *et al.*, 2016).

Nevertheless, in order to attain high PHA accumulations in PPB cultures, it is essential to enrich the culture in PHA-accumulating bacteria. There are different advantages and disadvantages to the aforementioned strategies when specifically addressing mixed PPB cultures, which will be discussed below.

4.IV.3. FEAST AND FAMINE (FF) STRATEGY

To select for PPB that stores PHA as carbon reserves, a FF strategy can be applied. Repeated FF cycles create a selection pressure that enriches the culture in organisms with high PHA storing capacity. Since this strategy requires the presence of an electron acceptor for the PHA consumption in the famine phase, Fradinho *et al.* (2013) proposed the operation of mixed cultures comprised of PPB and microalgae, with the latest being the oxygen providers. When the consortium is operated in a FF regime, the system is not completely anaerobic due to the oxygen production by microalgae, but it is under sub-oxic conditions. During the Feast phase, the oxidation and reduction potential (ORP) can drop to -300 mV, and PPB use the photosynthetically generated ATP to anaerobically take up external carbon and store it as PHA (Figure IV.2). In this situation, high carbon conversion efficiencies can be expected. During the famine phase, the cells' oxygen demand decreases, and the oxygen that is continuously produced by microalgae increases the ORP up to +0/+50 mV. Oxygen values are never detected, minimizing an eventual inhibition of PPB pigments expression.

Studies under these FF conditions and continuous illumination have shown the mixed cultures capability of accumulating up to 20% gPHA gVSS^{-1}, producing a PHA copolymer (HB:HV molar fraction of 84:16) when fed with VFA mixtures (Fradinho *et al.*, 2014). When the cultures were operated under dark/light cycles, microalgae levels could be decreased relatively to PPB,

further enriching the culture in PPB and enabling PHB contents up to 30% (Fradinho *et al.*, 2013). In this case, the PHB storage yield reached 0.90 ± 0.09 $Cmol_{PHB} Cmol_{Substrate}^{-1}$ (acetate + internal glycogen), confirming that high carbon recovery and conversion to PHA are possible during the feast phase.
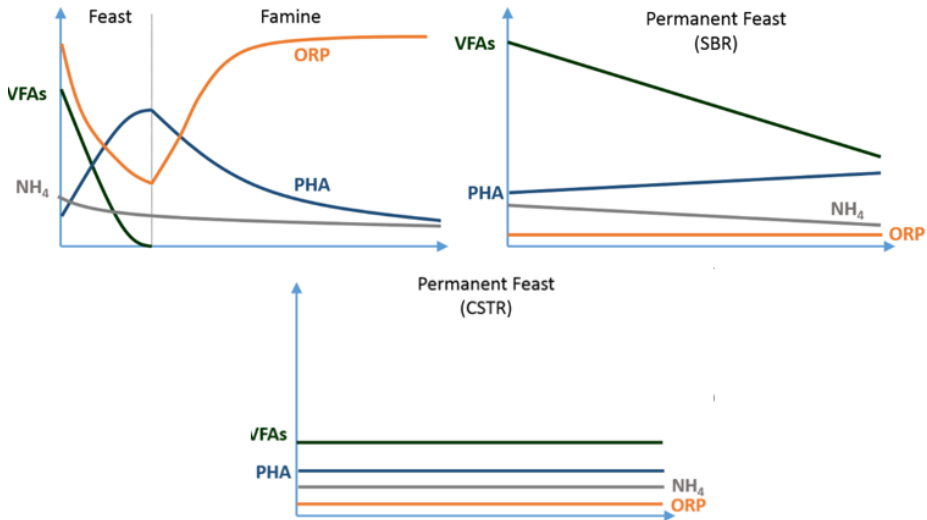
Figure IV.2 General profile of phototrophic mixed cultures during PHA production under different selection strategies and reactor operation. A: Feast and famine selection in an SBR; B: Permanent feast selection in an SBR; C: Permanent feast selection in a CSTR.

Overall, studies under the FF strategy indicate that it is possible to efficiently enrich the mixed cultures in PHA accumulating PPB by promoting PHA storage as carbon reserves. The cultures not only can produce PHA co-polymers when fed with SCCA mixtures (compounds commonly found in fermented organic wastes) but are also robust to transient illumination, an important feature for outdoor operation.

4.IV.4. PERMANENT FEAST (PF) STRATEGY

Another strategy that can be used to select for PHA accumulating PPB, explores the PHA storage as a sink for reduced power. Firstly, the PPB mixed culture must be cultured in an illuminated anaerobic environment under the permanent presence of SCCA (so-called permanent carbon feast, PF) (Fig. IV.2). In these conditions, PPB does not have access to the typical electron acceptors (oxygen, nitrate), and when they take up the organic carbon, PPB must activate metabolic pathways that allow electrons dissipation, thus balancing the cell's internal redox state. PHA accumulation, CO_2 fixation via Calvin-Benson-Bassam

(CBB) cycle, and N_2 fixation/H_2 production via nitrogenase enzyme are some metabolic processes that PPB can use to dissipate electrons (Alsiyabi *et al.*, 2019; McKinlay and Harwood, 2010). The key is to provide operating conditions that select for PPB that favor the PHA accumulation pathway. As such, if the PPB mixed culture is grown on feedstocks that contain NH_4^+ (which is usually the case in municipal wastewaters and some fermented organic wastes), nitrogenase expression is repressed, and electrons cannot be dissipated through N_2 fixation/H_2 production (Koku *et al.*, 2002). Regarding CO_2 fixation via the CBB cycle, this is an important pathway used by PPB to achieve redox homeostasis, especially when the cells are growing on organic substrates more reduced than the biomass (Koku *et al.*, 2002; McKinlay and Harwood, 2010). However, the CBB cycle is an ATP-dependent and high-energy-demanding pathway (Alsiyabi *et al.*, 2019). On the contrary, no ATP is required if reduced cofactors are re-oxidized via PHA production (Laycock *et al.*, 2014). With both pathways at hand, the organisms that favor electron dissipation via PHA production have more energy available, benefiting their growth and enriching the PPB mixed culture in PHA storing bacteria.

It is essential to point out that PHA production can occur side-by-side with cell growth with this PF selection strategy, with no detriment to the culture selection (Fradinho *et al.*, 2016, 2019). However, it is natural that more PHA can be stored if the carbon precursors are not being used for growth. This can be achieved by limiting growth (nutrient limitation) and/or increasing the light availability, allowing cells to take up more VFA than necessary to grow. This was observed in a study by Fradinho *et al.* (2016), where a PPB mixed culture selected under permanent acetate feast, P limitation, and low light availability could accumulate up to 60% PHB when exposed to higher light availability in accumulator reactors. Also, high PHB storage yields of 0.83 ± 0.07 $Cmol_{PHB}$ $Cmol_{Acetate}^{-1}$ and global carbon yields ($\sum Y$ accounting biomass + PHB + glycogen production) of 1.03 ± 0.05 Cmol $Cmol_{Acetate}^{-1}$ could be achieved (Fradinho *et al.*, 2019), further reinforcing the potential of PPB systems for high carbon recovery during PHA production.

The results show that targeting PHA storage as an electron sink is an attractive strategy that allows PPB mixed cultures enrichment in PHA storing PPB. Furthermore, in anaerobic conditions and PF the culture becomes fully enriched in PPB (algae are outcompeted). High PHA content and carbon recovery can then be achieved with light energy demands supported by natural sunlight illumination (Fradinho *et al.*, 2019).

4.IV.5. POTENTIALS FOR POLY-P PRODUCTION AS P RECOVERY

In the last decades, the world requirement for more pastures and crops for food production has increased the phosphorous demand, and since P is mostly obtained from finite phosphate rock mines, there are concerns that P rock reserves may become depleted (Solovchenko *et al.*, 2016). Furthermore, phosphate's widespread use and uncontrolled leakage into waterways can cause detrimental environmental problems, such as eutrophication, with profound negative effects on the ecosystems (Solovchenko *et al.*, 2016). Therefore, phosphorus recovery methods like the EBPR systems, have been implemented and widely used in wastewater treatment plants (WWTP) to recover phosphate from waste streams. In these systems, Poly-P storage requires intensive aeration (with associated costs), and part of the organic carbon removed from the wastewater is dissipated during PAOs' aerobic respiration. Implementation of photosynthetic processes can overcome these drawbacks, exploring, for example, the PPB capacity to store Poly-P, and designing specific systems to recover P anaerobically from waste liquid streams. Studies with PPB strains from *Rhodobacter sphaeroides* (Hirais *et al.*, 1991) and *Rhodopseudomonas palustris* (Liang *et al.*, 2010) showed that under illuminated anaerobic conditions and upon entering the stationary growth phase, these organisms accumulated substantial amounts of Poly-P. Unlike the chemoheterotrophic bacteria in EBPR systems that obtain energy by consuming organic carbon sources, ATP production in PPB is independent of carbon uptake. Therefore, with ATP constantly available from light, PPB could continue to take up phosphate, and because growth was limited, cells were no longer assimilating phosphate into biomass but instead storing it as Poly-P. These findings are even more interesting considering that *R. sphaeroides* could accumulate 2 – 4% of their cell dry weight as phosphorous, and *R. palustris* could achieve P contents of 4 – 10%. These values are in the range of P contents observed in PAOs, 5 -15% (Liang *et al.*, 2010), demonstrating the high storage potential of PPB.

In PPB mixed cultures, polyphosphate storage has also been reported, for instance, in cultures grown under P limiting conditions in a system aimed at PHA production (Fradinho *et al.*, 2016, 2019). In these studies, the mixed cultures were operated under low illumination conditions in the selector SBR. When exposed to high light availability, the phosphate uptake increased up to 95% of the removed phosphate accumulated and not assimilated into the biomass (Fradinho *et al.*, 2016). Despite the culture's previous conditioning to P starvation periods, the higher light supply in this study demonstrates the

importance of an excess of ATP availability to boost phosphate uptake and Poly-P storage.

The common finding between all these studies is that a surplus of ATP seems to be the critical factor that triggers high P storage. As such, a PPB system for P removal from wastewater can be devised by including periods with an excess of ATP availability. These conditions can be achieved by controlling several parameters like the light supply, which directly impacts ATP production, and by regulating the activity of some metabolic pathways that compete for ATP (e.g., ensuring the presence of ammonia to inhibit H_2 production). However, the hypothesis of combining poly-P storage with PHA production cannot be excluded if the operating conditions allow an excess of ATP capable of sustaining both phosphate and high carbon uptake. Indeed, simultaneous Poly-P formation and PHA storage were observed in (Fradinho *et al.*, 2016, 2019) with mixed and single strains of PPB, respectively. The prospect of implementing such a PPB system to waste streams processing would be very economically attractive and environmentally beneficial due to the concurrent P and C recovery and discharge of a better-quality effluent.

From the studies on P storage in PPB, Table IV.1 proposes a simple description of the different possibilities of producing Poly-P in combination (or not) with other compounds, assuming that cells have external P and ATP in excess for growth.

Table IV.1 Proposed combinations of Poly-P production with other compounds by PPB under different C, N, S cultivation conditions, assuming external P and ATP in excess for growth. TCA: Tricarboxylic acid cycle.

	High Carbon		Low Carbon or Low S
	VFAs	Organic acids (from TCA)	
NH$_4$ present	Poly-P + PHA (Fradinho *et al.*, 2016)	Poly-P (Hirais *et al.*, 1991)	Poly-P (Kitamura and Hiraishi, 1985)
NH$_4$ limited	Poly-P + PHA (Lai *et al.*, 2017)[1]	No Poly-P; likely H$_2$ (Kitamura and Hiraishi, 1985)	Likely Poly-P (no references)

[1] glutamate was used as N source

Mostly all the situations lead to Poly-P production, except for PPB growth in NH_4 limited conditions and the presence of TCA organic acids, a condition known for promoting H_2 production that competes with Poly-P for energy. A small note must be given regarding the Poly-P production with NH_4 presence and low carbon because, in this situation, it is fundamental to filtrate the visible light to prevent microalgae growth in such a nutrient-rich environment. With this filtration assured, PPB systems also become a potential technology for wastewater treatment and P recovery from streams with a low COD/P ratio.

4.IV.6. UPSCALING CHALLENGES AND RESEARCH NEEDS

At the current moment, a large amount of knowledge has been harnessed on PPB technologies, and these are starting to be implemented in outdoor pilot/demonstration conditions for a complete evaluation of their potential. However, this technology transfer from lower laboratory scales to larger facilities is usually associated with upscaling challenges that must be identified and tackled.

Regional limitations and challenges

As a light-dependent technology, large-scale operation of PPB systems must consider the conditions of the implementation site in terms of local climate (sunlight availability) and land requirement (enough space to accommodate the facilities). Insolation is not uniform around the globe, with some regions receiving higher irradiance levels than others, thus being more likely for implementing the photosynthetic process. Also, due to the Earth's tilt, the day length changes along the year, with the variations between day/night time becoming more intense with increasing latitudes. These seasonal variations will strongly impact the system operation during the winter periods and imply process adjustments (e.g., OLR, SRT) if an all-year operation is planned.

Local temperature is also an essential factor when selecting the operating site. The very high temperatures generally found in regions with the highest insolation may be excessive for PPB activity, preferably growing in the range of 25 to 35°C (Chen *et al.*, 2020). On the other hand, Hülsen *et al.* (2016a) reported that PPB cultures could adapt to lower operating temperatures (10°C) with performances comparable to temperate temperatures (22°C), which enlarges the regional applicability of PPB systems.

The implementation of PPB systems is very dependent on space availability. Like other photosynthetic processes, a high illuminated surface-to-volume ratio is fundamental to increasing productivity. While different reactor configurations have been proposed to increase this ratio (e.g., tubular reactors, flat panels), their high investments and maintenance costs are prohibitive for large waste streams applications (Gupta *et al.*, 2019). Open raceway ponds are a cheaper and simple alternative that can support the anaerobic growth of PPB mixed cultures, providing that organic carbon is present, which maintains the system under zero oxygen concentration levels. However, open ponds present liquid heights between 15 -30 cm, thus requiring a high ground surface area in order to process high volume loads. As such, their implementation is suited for the outskirts of small towns or nearby waste-producing industrial areas. This last case would be ideal for PPB systems since there would be (i) easy access to the waste stream, (ii) access to qualified technicians for the system operation, (iii) the possibility to integrate the PPB polymer production system with downstream and polymer processing units within the industrial zone, establishing a grid that follows the biorefinery concept. It is important to mention that the utilization of products recovered from waste streams is subject to national regulation. Therefore, effective communication between technology developers, companies, and political institutions is required to develop standards that ensure the quality and safety of the PPB technology and its products.

Bottlenecks in upscaling artificial light systems

Unlike sunlight, which has daily/seasonal irradiation variations and is affected by weather conditions, artificial light can be fully controlled in terms of irradiation time, intensity, wavelength, and spatial distribution. In the case of light wavelength, the utilization of LED lamps that emit in the near-infrared region (NIR) can even be used to selectively grow purple bacteria over microalgae (Puyol *et al.*, 2017). From a process point of view, artificial illumination would be the optimal light source for increased productivities of biomass and associated biopolymers (Fradinho *et al.*, 2019). However, it brings extra capital and operating costs from the economic side. Capson-Tojo *et al.* (2020) estimated an illumination cost of 1.9 $ per kg of PPB biomass grown under artificial LED illumination (based on 2019 US energy costs and assuming a maximum empirical value of 59 g COD kWh^{-1} for the biomass energy yield). In a biopolymer production process, which must also account for biomass downstream processing costs, these illumination costs would be an additional weight that likely could not be compensated from the incomes of PPB biopolymers produced from waste streams. Similar observations have been

made over the years concerning microalgae bioprocesses in which the utilization of artificial illumination is only advised for high-value molecules production (Blanken *et al.*, 2013). As such, free sunlight illumination is fundamental for the economic viability of PPB systems dedicated to the production of commodities, like PHA or fertilizers, which have lower commercial prices. Hence, PPB technologies should be designed in accordance with the constraints of solar irradiance and set up for outdoor operation.

4.IV.7. LIMITATIONS OF LIGHT IRRADIATION BASED ON RECTOR DESIGN AND POTENTIAL MEASURES TO SOLVE THESE LIMITATIONS

As stressed above, light irradiance is a key factor in developing the PPB bioprocess. These organisms can be cultivated via open or closed systems. Open raceways systems made of closed-loop recirculation channels are typically shallow (<0.3 m deep), unlined, and have a moderate surface-to-volume ratio of 3–10/m (Gupta *et al.*, 2019). These systems are also prone to contamination by pathogens and predators, experience water loss via evaporation, and low illumination efficiency. Nevertheless, they account for 95% of worldwide algal production (Mendoza *et al.*, 2013) due to the low capital and operation costs and easy scalability and operation. Closed systems or PBRs were designed to overcome the problems associated with open-pond systems, and culture growth in them is much more controlled. PBRs can be tilted at different angles using diffuse and reflected light that can play an important role in productivity. Although there are many examples of different PBR designs, two main types are commercially applied nowadays: tubular reactors and flat panel reactors. Both have already been heavily studied on PPB (Lu *et al.*, 2019), although mainly as indoor reactors. Regardless of the type of reactor used, sunlight is still the key limiting factor due to low light intensity, uneven distribution, shifts during the day/night cycle, changing weather conditions, seasonal changes, or direct exposure to UV irradiation. This light limitation does not allow fully exploring the growth potential of PPB and the production of associated polymers. Fradinho *et al.* (2016) reported specific growth rates values around 0.5-0.8 d^{-1} in PHA producing PPB mixed cultures selected under low light conditions. However, specific growth rates can range from 0.6 up to 12 d^{-1} in axenic cultures (Madigan and Gest, 1979). This variation naturally results from the diversity of PPB organisms tested and from the culturing conditions where temperature, carbon source, and light irradiation needs are known to impact the growth rates. For comparison purposes, in PHA producing aerobic mixed cultures, specific growth rates have been reported in the range of 0.96 to 2.16 d^{-1} (Oliveira *et al.*, 2017). However, in these systems,

culture selection occurs in the constant presence of oxygen (minimal oxygen saturation levels ~10%), supporting the culture's oxygen needs for ATP production and consequent growth. In photosynthetic systems, the difficulty in providing the ideal light input will limit ATP production and the capability to reach higher specific growth rates. This can explain why, in comparison to aerobic chemotrophic organisms, PPB systems typically present lower biomass production rates. As such, it is fundamental to develop new strategies to enhance solar illumination efficiency, exploring different solar collection and distribution systems, thermochromic materials or solar filtration technologies.

Solar collection and spatial light dilution systems

Spatial light dilution is a method to decrease photon flux density and distribute it in a larger surface area. This method requires diffusers and collectors such as optical fibers, parabolic discs, green solar collectors, or luminescent solar concentrator panels. Optical fibers are glass or plastic transparent fibers that can transport light from one place to another (for example, from a parabolic disc to inside the reactor). They have shown significantly increased productivities in microalgae cultivation and have already been studied on PPB, with a 1.38-fold increase in hydrogen production (Chen *et al.*, 2008). A breakthrough in new materials for the cost-effective production of optical fibers must occur since their excessive prices still make them unsuitable for large-scale applications. An exciting option is the use of poly-methyl-methacrylate as the primary material. This material has been proved efficient in increasing the phototrophic growth of the green algae *Haematococcus pluvialis* by around 95% (Wondraczek *et al.*, 2019). Interestingly, multi-wavelength light penetration through these optical fibers seems to be better in the near-infrared range, so it is a promising material for being used with PPB. However, biofilm formation over the optical fiber is a major drawback for continuous operation if the process is focused on biopolymers production, which considerably limits the volumetric productivity. A potential solution for this is applying a smart low-voltage electric field to avoid bacterial adhesion over the illuminated surface. This has been successfully applied to avoid microbial fouling problems in MBR reactors (J. Zhang *et al.*, 2015).

The most promising spatial dilution method is luminescent solar concentration panels, built by luminescent particles like organic dyes or quantum dots that absorb and re-emit light at longer wavelengths (Hill *et al.*, 2019). The use of luminescent solar concentrations has already been tested on raceway reactors for microalgae and cyanobacteria cultivation, with

Raeisossadati *et al.* (2019) finding a 26% increase in biomass productivity for *Arthrospira platensis*. Luminescent solar concentrations are easy to construct, cost-effective, and feasible to use in outdoor open pond systems, but they must be tested on PPB since the wavelengths needed to improve productivity differ from microalgae cyanobacteria.

Solar filtration technologies

The development of wavelength filtration systems has allowed the specialized culture of PPB using only IR or NIR wavelengths. The more typically used method in laboratory-scale PPB cultivation is UV-VIS absorbing foil, but even though it is a cheap material, its use in outdoors large-scale reactors does not seem suitable since it is an easily tear-off material. Another option is colored filter glass, a relatively inexpensive filter type that attenuates light but sacrifices absorption. The design of PBR with this material has already been tested, improving productivity in microalgae (Sun *et al.*, 2018). Temperature upsurge due to the absorbed wavelengths is still the biggest drawback, thus making it less adequate under intense illumination conditions. Finally, thin-film coatings destructively interfere with unwanted wavelengths instead of absorbing them, solving at the same time the overheating problems of glass coatings. These materials can easily be adapted for a PBR design, and they were recently tested on a cascade photobioreactor to cultivate high-density microalgal cultures for biodiesel production (Tan *et al.*, 2020). They have not been proven for PPB growth yet, but it has the potential to be a cost-efficient technology.

Thermochromic materials

One of the biggest problems in outdoor reactors is seasonal temperature changes. Specifically, in winter, low temperatures can cause a decrease in metabolic efficiency and even freeze the reactor. Thermochromics materials can change their transmittance and reflectance properties when subjected to temperature variations. They are usually employed as glazing materials in architecture, thus regulating the amount of light transmitted or reflected depending on atmospheric temperature (Kamalisarvestani *et al.*, 2013). They act as specific wavelength filters and transmit more heat associated with IR and near-IR radiations at low temperatures (Granqvist and Niklasson, 2017). On the other hand, they reflect IR wavelengths at high temperatures, so they should be adapted to the reactor and easily removed during hot days. These properties can be perfectly adapted to the needs of PPB cultivation through effective sunlight wavelength management and temperature regulation, but it needs an

exhaustive experimental study to determine its suitability. No work has been published so far applying this technology.

4.IV.8. PPB RETENTION SYSTEMS FOR BIOPOLYMERS PRODUCTION AND POTENTIAL MEASURES TO SOLVE BOTTLENECKS

PPB can be cultivated in reactors/tanks with varied configurations, where cells can freely circulate in the system or be retained through the presence of carriers, support materials, or membranes (Chen *et al.*, 2020). Biomass retention systems have been proposed to reduce the system volume by concentrating cells, improving nutrient removal from the liquid stream, and facilitating liquid separation from the biomass (Chen *et al.*, 2020). Cells can be retained, for example, by using photo-anaerobic membrane bioreactors (PAnMBR) as proposed by Hülsen *et al.* (2016b) for wastewater treatment with PPB. However, when PPB are operated for polymer production, the goal is quite the opposite of biomass retention. Instead, the aim is to remove as much biomass as possible for stable polymer extraction. Therefore, it is even more critical for up-scaled systems that simple and direct access to the biomass is implemented with the lowest possible manual labor requirement. At the current technology development, suspended cells systems may be a better option, and while some settling issues may be pointed out, they may be minimized by the capacity of PPB to attach to particles present in the waste stream, forming granules. This can improve sludge settling and subsequent biomass recovery in PPB mixed culture systems.

Settling

One of the highest operational costs in polymer extraction is the dewatering of biomass after its cultivation (Alloul *et al.*, 2018). Settling is one of the most established methods, but PPB collection is challenging due to their small size (0.2-4 µm), high electronegativity, and stable suspension state in the culture medium (Chen *et al.*, 2020). The easiest and cheapest would be gravitational settling, but if not possible, membrane filtration can be performed followed by centrifugation (Alloul *et al.*, 2018), although it will increase operating costs. Recently, Cerruti *et al.* (2020) developed an enriched, concentrated and well-settling PPB mixed-culture operated under the SBR regime. This mixed-culture forms bio-aggregates with good settling properties. Settled biomass accounted for a 3-fold higher relative abundance of PPB (80% as the sum of *Rhodobacter*, *Rhodopseudomonas*, and *Blastochloris* sp.) than the non-settled biomass (25%) with sedimentation G-flux of solids up to 4.7 kg h^{-1}

m^{-2}. This should lead to an efficient solid to liquid separation, lowering costs for downstream processing by potentially reducing the need for ultrafiltration and centrifugation to concentrate the biomass.

Flocculation

Generally, flocculation has some advantages for industrial processes because of the simplicity of liquid/solid separation and the ease of mass cell retention in the reactor. However, flocculating photosynthetic bacteria have not yet been practically applied for industrial use due to their high cost associated. Some studies demonstrate how sodium, pH, and light intensity affect flocculation for *Rhodobacter sphaeroides* (Lu *et al.*, 2019). Watanabe *et al.* (1998) studied the growth and flocculation of *Rhodovulum* sp and tried to improve it by adding metal cations. The only study found about flocculation on PPB mixed cultures was published in 1983 and uses aluminum sulfate as a flocculant (Freedman *et al.*, 1983). So, this is an open field of research in this process with PPB mixed cultures.

Photo-Granulation

From a bioprocess perspective, the high potential of granules to settle facilitates the recovery and reuse of the active microbial biomass, and in the wastewater treatment context, the development of oxygenic photo-granules is a good novelty (Abouhend *et al.*, 2018). Also, granules can give way to IR light photons better than suspended biomass. Following the Beer-Lambert law, light absorbance will be enhanced with concentrated biomass, making deeper bioreactors possible, improving volumetric productivity and reducing economic costs. Most studies have been performed on microalgae, but the research on other microorganisms is attracting increased interest, and PPB's ability to form granules has been proven (Stegman *et al.*, 2021; Wilbanks *et al.*, 2014). In Section III, we have already shown that the formation of EPS granules directly affects PHA accumulation. Controlling the formation of photo granules is an open invitation to interdisciplinary collaborations between ecologists, bioprocess engineers, and environmental scientists.

4.IV.9. HOMOGENEITY OF THE OPERATION

Mixing is a crucial feature in the cultivation of any photosynthetic microorganism. Homogeneous mixing can reduce the gradient of nutrients, pH, temperature, and substrate in the reactor, preventing biomass settling, stagnant or dead zones, and cell aggregation (Kumar *et al.*, 2015). Besides,

homogeneity ensures that all cells are equally exposed to the light and promotes mass transfer between phases. However, mixing accounts for approximately 69% of the total utility cost, in the range of 1.5–8.4 W m^{-3} (Mendoza *et al.*, 2013). Therefore, unnecessarily mixing should be avoided, reducing the mixing velocity during the night and even in the winter season to minimize the operational cost. On the positive side, power consumption can be minimized under lower volume depths, which agrees well with PPB cultivation, and deteriorates its growth over 20 cm of depth due to restricted illumination.

Several modifications in PBR have been proposed to tackle mixing costs and demonstrated to enhance biomass productivity, mixing efficiency, and light penetration, such as closed ponds, hybrid raceways, different flows through modular stack raceways, or unlevelled baffled raceways (Kumar *et al.*, 2015). Another vital point to consider is the inlets and outlet points on the reactor. A single inlet point can create poor mixing and substrate gradient throughout the reactor (Enfors *et al.*, 2001). Thus, multiple inputs to the PBR should be considered, even more so in larger reactors.

4.IV.10.   AUTOMATIZATION OF THE SYSTEM

Control systems play an essential role in the development of bioreactors. In general, the main functions of a bioreactor control system include process control, monitoring, data gathering, and processing. The current trends in bioreactor control systems can be grouped into two aspects: some studies are focused on control strategies and algorithms adapted to bioreactor control systems (Steinwandter *et al.*, 2019), while others mainly investigate the physical structure of the instrumental organization of bioreactor control systems (Wang *et al.*, 2020).

PPB activity can be assessed by key performance indicators such as pH, absorbance, temperature, illumination, nutrient and substrate recovery rates, and biomass productivity, for which suspended solids and nutrient concentrations must be measured. Although online sensors can monitor ammonium, nitrate, and suspended solids concentrations, they usually have higher capital and maintenance costs but are not always as reliable as expected. Therefore, they are often measured by time-consuming and expensive laboratory analyses (Foladori *et al.*, 2018). The same happens with the production of biopolymers since their intracellular concentration can only be measured in the laboratory, which amplifies the need for online monitoring strategies based on dynamic modeling dependent on indirect parameters such

as pH, absorbances at 805 and 8065 nm, illumination, temperature, and the hydraulic retention time. But the complexity, inherent nonlinearity, and high kinetic uncertainty can make this approach very complicated. There are recent examples to control microalgae activity, such as the works published by De-Luca *et al.* (2018), where a growth optimization based on uncertain weather forecasts is shown, or Robles *et al.* (2020), which demonstrated a community stabilization based on pH and dissolved oxygen. However, no work on the control of PPB activity has been published yet. Control systems should be implemented as part of a SCADA (Supervisory, Control, and Data Acquisition) program for supervision, data acquisition, and equipment control. Reactor level control, feeding times, mixing, among others, can be output signals that can allow for better system optimization upon the dynamic model algorithm implementation.

4.IV.11.   FUTURE DIRECTIONS ON PHA PRODUCTION WITH PPB MIXED CULTURES

In prospecting the transfer of the PHA producing technology with PPB mixed cultures to larger-scale outdoor facilities, it is essential to mention that the two selection strategies currently applied to enrich the mixed culture in PHA-producing PPB, FF and PF, have very distinct modes of operation and requirements. Both strategies present solid points but also aspects that can be further improved. Table IV.2 compares, side by side, the main characteristics of the two strategies.

Looking at the characteristics of the FF strategy, the major factor that can hinder the technology transfer to large outdoor operations is the system dependence on microalgae to produce the oxygen that enables the PHA consumption during the famine phase. There is, however, margin to develop the FF technology by making the system independent of the microalgae presence. The microalgae elimination could further enrich the mixed culture in PPB while the advantageous features of the FF operation could be maintained. As previously mentioned, the system requires an electron acceptor during the famine phase, which can be provided through external aeration. Although it brings additional energy costs to the famine phase, the oxygen needs during the famine phase are substantially lower than the feast phase (2 − 5 times lower) (Third *et al.*, 2003), and thus minimal aeration would be required.

Table IV.2 Characteristics and specific requirements of the feast and famine and the permanent feast strategies used for the selection of PHA producing PPB mixed cultures.

	Feast and Famine (PPB and microalgae)	Permanent Feast (PPB enriched culture)
Aeration	Aeration is eliminated since the microalgae provide auto-oxygenation; FF cycle must occur during the daytime.	Anaerobic operation, no aeration requirements.
Carbon recovery	High carbon recovery and conversion to PHA is achieved during the feast phase; PHA is partly dissipated as CO_2 during the famine phase.	Up to 100% carbon recovery (approaching the system to CO_2 neutrality) with high conversion to PHA.
Nutrient limitation	Carbon is the limiting nutrient that triggers PHA storage; No other nutrient limitation is required.	High PHA content is achieved under nutrient limitation, requiring feedstock with high carbon to nutrient ratios (e.g., high C:P or C:S ratios)
Electron balance	Oxygen availability during the famine phase reduces PPB internal redox stress allowing the uptake of more reduced substrates during the feast phase (e.g., butyric and valeric acids).	Anaerobic conditions induce consumption of less reduced substrates (e.g., acetate, propionate) to facilitate redox balance, leading to the accumulation of less preferable compounds
OLR	Strict control in order to maintain a stable feast to famine ratio.	No famine phases; Organic carbon is continuously present, simplifying system operation
Sugar presence in the feedstock	Oxygen presence inhibits fermentative metabolism.	Full fermentation of the feedstock must be assured to prevent fermentation processes that conflict with PHA production

Presence of Non-PHA producers	Although essential for oxygenation, microalgae decrease the overall PHA content in the biomass and may compete for carbon (e.g., acetate);	Microalgae are outcompeted; Fermenting organisms may grow if fermentable compounds are present in the feedstock (e.g., sugars, lactate)

Therefore, future studies should evaluate if the costs incurred with aeration in the famine phase could be overcome by the following advantages: (i) the famine aerated phase could be schedule to the night time, and thus, the night would no longer be an idle period, but instead, could actively contribute to the FF process; (ii) with the famine phase occurring in the night, the feast phase could be extended up to the night time increasing the system PHA productivity and eventually eliminate the need for a secondary accumulator tank reactor (iii) aeration during the night famine phase allows the consumption of residual organic compounds that are not assimilated by PPB during the day anaerobic feast phase, permitting the discharge of a better quality effluent, (iv) aeration rates can be easily tuned to address the culture oxygen requirements at each night famine phase, maintaining the culture under very low oxygen concentrations (0.0-1.0 mgO_2 L^{-1}) which decreases aeration energy costs and minimizes inhibition of PPB pigments expression, (v) the operational conditions lead to a microbial selection that favours the mixed culture enrichment in PPB that are more oxygen tolerant, which further overcomes eventual oxygen inhibitions due to the night aerated time. With these adaptations, the FF strategy would be ideal for operation with a feedstock with highly reduced carbon compounds and high nutrients since it does not rely upon nutrient limitation for improved PHA accumulation. However, carbon dissipation will always occur in the famine phase during PHA consumption.

Concerning the PF strategy, its major challenge is related precisely to the feedstock composition. Since this strategy relies on the observance of anaerobic conditions, the presence of sugars and high sulfate concentrations in the feedstock can lead to the growth of a side-population composed of fermentative organisms or even of sulfate-reducing bacteria (the latest supported by the highly reductive conditions promoted by the sugars). Therefore, it is essential to control the sugar present in the feedstock by implementing, for example, upstream fermentation processes. Furthermore, high nutrient concentrations in the feedstock can also challenge the production of high PHA content. In this situation, to favor the PHA storage over the growth, additional metabolic constraints may be needed in parallel to the selection under the permanent feast strategy. For example, adjusting the ratio of food to microorganisms (F/M) may be a possible solution since F/M has been

suggested to impact the dynamics of internal carbon flow during PHA storage in PPB systems (Fradinho *et al.*, 2016). Also, eventual growth decouplers (aromatics, metals) in the feedstock may help limit growth but not the PHA production activity (Puyol *et al.*, 2019). Therefore, the PF selection strategy is suited for operation with a feedstock that contains less reduced carbon sources (e.g., acetate, propionate) and low nutrient concentration (P, S) that allow the PHA storage over the growth. Compared to the FF strategy, it has the advantage of high carbon recovery that approaches PF systems to CO_2 emission neutrality.

For the outdoor operation of PPB mixed cultures under the PF selection strategy, the current knowledge proposes the operation of two reactors/tanks: one for culture selection and growth with low light availability and a second for an external accumulation step under higher light availability. Decreasing the implementation area through one single reactor operation would be a more economically attractive alternative. This raises the interesting and not yet addressed question of whether simultaneous culture selection and high PHA production are possible. The culture would permanently present high intrinsic levels of PHA, but this would imply the system operation with higher light availability. A possible conflict with the culture selection could occur (as previously discussed). However, a decrease in the culture's overall photosynthetic efficiency could be observed (reduction of light-harvesting complex levels due to acclimation to high light operation) (Muzziotti *et al.*, 2017). Further research can bring more insight to this challenging question.

4.IV.12. CONCLUSIONS

The sustainable production of biopolymers from waste sources by mixed cultures of PPB is an emerging field facing several challenges. Using current knowledge on mixed outdoor reactors based on PPB mixed cultures systems is crucial for achieving full-scale deployment of the technology. In addition, the need to advance on upscaling challenges like improved sun illumination, biomass retention and collection, and feed control has promoted the development of specific projects focused on these challenges.

4.IV.13.   REFERENCES

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CHAPTER 5:
CONCLUSIONS AND
PERSPECTIVE

5.1   GENERAL CONCLUSIONS

Although specific conclusions have been included in each Section of the results and discussion Chapter, the following is answers to the questions stated Chapter 2 of this present Doctoral Thesis.

1)   Is it possible to grow a mixed culture of PPB and accumulate PHA using OFMSW hydrolysates?

A mixed culture of PPB has been successfully cultivated from the liquid fraction of OFMSW hydrolysate, both pre-sorted by citizens and selectively sorted at the waste plant. PPB growth has been limited by the availability of nutrients, not by the biodegradability of organic matter. However, this nutrient limitation stimulates the accumulation of PHA from the PPBs, achieving a result of 5% wt. Furthermore, even with low nitrogen concentrations in the substrate, the PPBs contained 48% wt of protein which can also be seen as a high value-added product to be considered. The strategy of concatenating thermal hydrolysis and anaerobic digestion with a photoheterotrophic process results in high value-added products, but also reduces the volume of remaining solids by 5-11% compared with the traditional treatment of an anaerobic digestion process.

2)   How does using a carbohydrate-based residue such as lignocellulosic waste affect the PHA accumulation by PPB?

Using the same strategy, mixed cultures of PPB were successfully cultivated from liquid LW hydrolysates. Although these hydrolysates are mainly composed of sugars, PPB managed to assimilate them and also to accumulate PHA (21% wt.). Likewise, as with OFMSW, PPB grows from this organic matter without inhibition, and growth is limited by nutrient consumption. This Thesis is the first time that PHA accumulation is studied with LW, which allows to open this technology to agri-food sectors and to study synergies within classical biorefineries.

3)   How do high percentages of lignocellulosic residue affect the acidogenic co-fermentation of the OFMSW?

Given that PPB has a higher capacity to accumulate PHA using SCCA as substrate, this Thesis optimized the acidogenic fermentation process, evaluating whether there is synergy in the co-fermentation of FW and LW wastes. First, the combination of steam explosion and acidogenic fermentation achieves COD solubilizations of up to 80%, even with high percentages of LW.

Under the best-tested conditions, co-fermentation produced up to $162\pm5\,\mathrm{mlH_2}$ $\mathrm{gCOD^{-1}}$. H_2 production decreased notably with the increase of LW, the effect being stronger in the absence of thermal treatment. However, the fraction of COD derived to H_2 is very low (below 4%), and co-fermentation increases SCCA production which translates into a positive synergy effect in the overall efficiency. Values up to 0.58 gCOD of SCCA per gCOD added were achieved with coupled steam explosion and acidogenic fermentation of mixed urban waste, resulting in 93% acidification. Overall, the co-fermentation of FW and LW produces positive synergies, enabling this technology to be used on OFMSW with high LW content or even wastes from agricultural sectors. The results of this Thesis demonstrate the robustness of the acidogenic fermentation technology, which is not only key to optimize the photobiorefinery proposed in this Thesis but can also be used within the context of the carboxylate platform for a wide range of applications such as the biological removal of nitrogen and phosphorous from wastewater, biodiesel production or chain-elongation technology, increasing its versatility.

4) Is it possible, and which parameters are key to maintain a good PHA productivity from OFMSW fermentate?

This Thesis studied the continuous accumulation of PHA in a novel MPBR and obtained the best results of PHA accumulation with mixed PPB cultures and residual substrate in literature (42% wt), achieving a mass balance of the overall process of 0.15 $\mathrm{gCOD_{PHA}}$ gCOD which is in the same order of magnitude as the aerobic chemotrophic feast and famine processes, which are much more optimized and researched processes. Besides, the quantification of PHH, in addition to the usual PHB and PHV, represented up to 30% of the total PHA at certain stages, which increased the cumulative PHA identified. On the other hand, increasing the organic load of the reactor resulted in a decrease in PHA but an increase in glycogen, EPS and H_2 which shows, for the first time, alternatives to PHA accumulation in PPB mixed cultures: the storage of carbon in both glycogen and extracellular polymers (EPS) while deriving the excess electrons into hydrogen even in the presence of organic ammonium. Furthermore, this Thesis showed the need to study in-depth the functionalities of each bacterial community within mixed cultures of PPB. This Thesis demonstrated the importance of acquiring a thorough understanding of the carbon accumulation and electron allocation strategies of PPB under stressful

environmental conditions and shows promising results for a larger scale implementation of a PPB-based photo-biorefinery.

5)   Is the anaerobic digestion process suitable to close the biorefinery's carbon cycle and energy balance?

Anaerobic digestion has proven to be an adaptable and mature technology for the treatment of the non-hydrolyzed organic matter. Hydrothermal pretreatment of LW affects the overall biodegradability more positively than that of OFMSW, since the methanogenic potential is improved after pretreatment even when the liquid fraction is removed (contrary to OFMSW). The methanogenic potential of the organic fraction of the acidogenic fermentation effluent obtained high results (336 LCH_4 kgVS^{-1}), reducing the volume of solids of the integrated photobiorefinery by up to 90% in total. The energy produced in a CHP fed with biogas from anaerobic digestion is sufficient to satisfy the energy requirements of the steam explosion pretreatment and the heat required in the biodigester. This energy autarky is key to achieving a cost-effective integrated photobiorefinery.

6)   What are the major challenges in scaling up this technology?

The major challenge of scaling up the photoheterotrophic process with mixed PPB cultures is the design of a bioreactor that can maximize the volumetric irradiance received, without being critically reduced by increasing the biomass concentration. In the short term, the most viable solutions are to place these reactors in regions with high solar irradiance throughout the year, as well as to install solar collectors that increase the irradiance fed back into the reactor. Closed reactors would be ideal to avoid contamination of other communities (microalgae) and to ensure an anaerobic environment, but open raceway reactors would be more economical and IR light filters could drastically reduce contamination. Scaling up the system to a permanent feast operation would also streamline the process to a single reactor, thereby reducing costs. Research is in the sweet spot for higher TRL PPB-based integrated photobiorefineries, from which rawn conclusions to eventually achieve industrial development.

5.2 RECOMMENDATIONS FOR THE FUTURE

Based on the results of this Thesis, and on research currently being carried out by other research groups, future directions in PPB culture research, are discussed below:

▪ **Upscale research**. Some guidelines for future research on PHA accumulation with PPB crops are already discussed in Section IV. Upscaling this technology is the hotspot of the research that is taking place now and probably in the years to come. This upscale will be performed most likely in raceway bioreactors with either solar or solar plus artificial lighting. The strategy most likely to succeed is the permanent feast, which has much higher carbon recovery, but raises the yet unresearched question of whether culture selection and high PHA production can be maintained simultaneously. Recent research indicates that the overall PHA production may decrease due to the acclimatization of the culture after light stress (Bayon-Vicente *et al.*, 2020). But more research is needed in this respect.

▪ **Nutrient decoupling.** In this Thesis, primarily substrates with a very high COD/nutrient ratio have been used. This nutrient limitation is critical for the PPB culture to accumulate PHA but restricts growth and therefore limits the volumetric productivities of the reactor. Therefore, decoupling nutrient limitation could be achieved by first allowing a high nutrient content to maximize growth and then limiting the nutrient content to maximize PHA accumulation. This is reasonable, as waste such as OFMSW or lignocellulosic waste can be mixed with, e.g., domestic wastewater, which has a high nutrient content.

▪ **Modeling the metabolism of PPB towards PHA production.** One key challenge is the integration of the complex metabolism of these organisms to allow control and optimization, which needs a dedicated modeling approach with novel components, including energy, electrons, and light harvesting complex. Due to the extraordinarily complex metabolism of PPB, the quest for a comprehensive and usable model is challenging as it should be stoichiometrically correct (thereby should be based on metabolic mechanisms) and easy to be implemented in real plants. Unfortunately, most of the models published so far address the identification of metabolic mechanisms of PPB from a systems biology perspective (including energy, carbon, and redox flows) and are not suited to analyze the optimal process conditions to produce biopolymers without major simplifications.

▪ **Granulation of PPB cultures.** In recent years, some research has been done on this aspect (Stegman *et al.*, 2021), but mainly focused on the recovery of wastewater resources and not on the production of high value-added products. As discussed in chapters 3 and 4, fractionation can reduce costs in the downstream process. Furthermore, optimization and versatility based on biopolymer accumulation (PHA, glycogen, and EPS) should be studied. Furthermore, H_2 production with this type of technology has not yet been studied.

▪ **Combination of catalytic and biotechnological pretreatments**. Especially considering lignocellulosic waste, a large part of the organic carbon is not being fully exploited, catalytic treatments either in situ or ex situ can be explored. For example, thermo-catalytic treatment prior to fermentation can reduce the necessity of hydrolysis and therefore improve the overall efficiency of the process (Ventura *et al.*, 2021).

▪ **Microbial protein production**. Microbial protein is also a high added value product, and thus many studies on the valorization of organic waste by biotechnological methods, including PPB, are turning towards this strategy (Alloul *et al.*, 2019; Hülsen *et al.*, 2020). It is important to mention that PHA increases the value of the microbial protein as it increases its probiotic quality. In any case, a photobiorefinery capable of adapting to produce different products according to seasonality or the type of substrate received in the plant would be an immense advantage. To this end, studies on process optimization and synergies in the downstream are still pending.

5.3   REFERENCES

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