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TESIS DOCTORAL

TURNING WASTE INTO VALUE: PRODUCTION OF THE HIGH-VALUE COMPOUNDS POLY(3-HYDROXYBUTYRATE) AND LACTIC ACID FROM RENEWABLE FEEDSTOCKS BASED ON MUNICIPAL SOLID WASTE

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CONTRIBUCIÓN DEL AUTOR

Yo Jon Kepa Izaguirre Campoverde, declaro que:

Esta Tesis Doctoral ha generado tres artículos (manuscritos 1, 2 y 4), los cuales han sido publicados en revistas indexadas JCR de primer y segundo cuartil. Además, el manuscrito 3, que aún no se ha publicado, será enviado a la revista Bioresources Technology.

- Izaguirre, J.K., da Fonseca, M.M.R., Fernandes, P., Villarán, M.C., Castañón, S., Cesário, M.T., 2019a. Upgrading the organic fraction of municipal solid waste to poly(3-hydroxybutyrate). Bioresour. Technol. 290, 121785. https://doi.org/10.1016/j.biortech.2019.121785 (ver manuscrito 1).

- Izaguirre, J.K., Manuela, M.R., Castañón, S., Villarán, M.C., Cesário, M.T., 2020. Giving credit to residual bioresources: From municipal solid waste hydrolysate and waste plum juice to poly (3-hydroxybutyrate). Waste Manag. 118, 534–540. https://doi.org/10.1016/j.wasman.2020.09.014 (Ver manuscrito 2)

- Izaguirre, J.K., Dietrich, T., Villarán, M.C., Castañón, S., 2019b. Protein hydrolysate from organic fraction of municipal solid waste compost as nitrogen source to produce lactic acid by *Lactobacillus fermentum* ATCC 9338 and *Lactobacillus plantarum* NCIMB 8826. Process Biochem. https://doi.org/10.1016/J.PROCBIO.2019.09.028 (ver manuscrito 4).

Por otro lado, se ha publicado un artículo científico adicional, fruto de la estancia internacional realizada en el Instituto Superior Técnico de Lisboa y que está relacionado con la presente Tesis Doctoral (ver manuscrito 5).

- Tůma, S., Izaguirre, J.K., Bondar, M., Marques, M.M., Fernandes, P., da

Fonseca, M.M.R., Cesário, M.T., 2020. Upgrading end-of-line residues of the red seaweed *Gelidium sesquipedale* to polyhydroxyalkanoates using *Halomonas boliviensis*. Biotechnol. Reports 27. https://doi.org/10.1016/j.btre.2020.e00491

La participación de varios investigadores como co-autores de los artículos publicados aquí, evidencia que el presente trabajo es el resultado de una colaboración dinámica entre Neiker y otros centros de investigación, como el Instituto Superior Técnico de Lisboa y Tecnalia.

Así mismo, a partir de los resultados obtenidos en los diferentes estudios que componen este trabajo, se han presentado a congresos las siguientes comunicaciones:

- Urreta, I., Suarez-Alvarez, S., Munarriz, S. N. M., Izaguirre, J. K., Castañón, S., Recycling organic residues into valuable biomass through the heterotrophic culture of *Chlorella protothecoides*. 7th International conference on algal biomass, biofuels and bioproducts (EEUU, Washington., June 2017).

- Cesário, M.T., Izaguirre, J.K., Marques, M., Fernandes, P., da Fonseca, M. M. R., Seaweed, a source of carbohydrates for the biological production of polyhydroxyalkanoates. European Symposium on Biochemical Engineering Sciences (Portugal, Lisbon., September 2018).

- Izaguirre, J. K., Urreta, I., Suarez, S., Castañón, S. Municipal solid waste as a sustainable bioresource to produce culture media. The 7th International Conference on Engineering for Waste and Biomass Valorization (Chez Republic, Prague., July 2018).

- da Fonseca, M. M. R., Izaguirre, J. K., Marques, M., Fernandes, P., Cesário, M. T. A quest for abundant and sustainable carbon sources in polyhydroxyalcanoates production – Are seaweeds the answer? 18th European Congress on Biotechnology. (Switzerland, Geneva., July 2018).

- Marques, M. M., Izaguirre, J. K., Fernandes, P. C., da Fonseca M. M. R., Cesáreo, M. T. Seaweed, a platform for the production of biodegradable bioplastics. 7th International Seaweed Conference (Ireland, Galway., November 2018).

- Izaguirre, J. K., da Fonseca M. M. R., Fernandes, P., Villarán, M. C., Castañón, S., Suarez, S., Cesário, M.T. Assessing the Organic Fraction of Municipal Solid Waste as Carbon Source for Poly(3-hydroxybutyrate) Production. 4th EuCheMS Conference on Green and Sustainable Chemistry (Spain, Tarragona., September 2019).

- Cesário, M. T., Izaguirre, J. K., Castañón, S., da Fonseca, M. M. R. Municipal solid waste as a feedstock for P(3HB) production. 10th European Symposium on Biopolymers (Germany, Straubing., September 2019).

RESUMEN

El modelo de sociedad actual y el aumento de tamaño de las ciudades, está generando un incremento de la generación de residuos sólidos municipales. Este hecho ha puesto de relieve un problema que no solo afecta la parte medioambiental, sino que también la económica y la social. Por consiguiente, su gestión se ha convertido en un reto para el cual se están buscando soluciones efectivas e innovadoras.

En este trabajo, bajo un enfoque de bioeconomía circular, se estudió la posibilidad de utilizar la fracción orgánica de residuo sólido urbano, así como el compost derivado de esta, como materias primas para la producción de poli(3-hidroxibutirato) y ácido láctico, respectivamente.

Por un lado, la producción de poli(3-hidroxibutirato) se llevó a cabo mediante el cultivo, en medio líquido, de la cepa *Burkholderia sacchari* DSM 17165. Para ello, se empleó como medio de cultivo un hidrolizado rico en azúcares obtenido a partir de la fracción orgánica de residuo sólido urbano. La obtención del hidrolizado se realizó mediante la combinación de un pretratamiento termoquímico y una hidrólisis enzimática. El hidrolizado resultante fue adecuado para el crecimiento de la cepa, pero el ratio C/N no fue lo suficientemente alto como para que tuviera lugar la producción de polímero. No obstante, la suplementación del hidrolizado con un extra de glucosa y sales incrementó el ratio y suplió los requerimientos nutricionales del microorganismo, dando lugar a la producción de poli(3-hidroxibutirato). Así mismo, con los datos obtenidos en este primer estudio, se llevó a cabo el escalado del proceso de producción del biopolímero a escala de laboratorio (5 L). Para ello se utilizó el hidrolizado de la fracción orgánica de residuo sólido urbano como medio de cultivo inicial (fase batch) por su equilibrio nutricional y un concentrado de residuos de ciruela, rico en azúcares, como “feed” en la fase fed-batch. Finalmente, se evaluó la viabilidad tecno-económica del proceso mediante una simulación con el software SuperPro Designer®. Los resultados obtenidos indicaron que, aunque la producción

de poli(3-hidroxibutirato) es técnicamente viable, no lo es económicamente, debido principalmente al bajo rendimiento de la fermentación.

Por otro lado, con una metodología similar a la anterior, se diseñó una estrategia para la obtención de un concentrado de aminoácidos a partir del excedente de producción de compost generado con la fracción orgánica de residuo sólido urbano. Dicha estrategia consistió en una hidrólisis proteica utilizando una combinación de varios enzimas. El hidrolizado obtenido en esta primera etapa, se complementó con sales y se testó como fuente de nitrógeno para la producción fermentativa de ácido láctico por *Lactobacillus fermentum* ATCC 9338 y *Lactobacillus plantarum* NCIMB 8826, logrando concentraciones de ácido láctico cercanas a 10 $g \cdot L^{-1}$. A pesar de que los ensayos se realizaron en matraz y de que es necesario realizar estudios a escalas mayores, los resultados obtenidos son prometedores y suponen un buen punto de partida para desarrollar métodos de producción de ácido láctico más sostenibles y con un menor coste económico.

La innovación de estos enfoques radicó en explotar la alta generación de residuos para la producción de compuestos de alto valor, contribuyendo de esta manera a la creación de escenarios de economía circular en torno a los mismos.

ABSTRACT

Today's societal model and the growth in size of cities are both increasing the generation of municipal solid waste. This fact has highlighted a problem that not only affects the environment, but also the economy and society. Consequently, its management has become a challenge for which effective and innovative solutions are being sought.

In this work, in a circular bioeconomy approach, the possibility of using the organic fraction of urban solid waste, as well as the compost derived from it, as materials for the production of poly(3-hydroxybutyrate) and lactic acid, respectively, was studied.

On the one hand, the production of poly(3-hydroxybutyrate) was carried out by cultivating, in liquid medium, the strain *Burkholderia sacchari* DSM 17165. To this end, a sugar-rich hydrolysate obtained from the organic fraction of municipal solid waste was used. The hydrolysate was obtained by combining a thermochemical pre-treatment and an enzymatic hydrolysis. The resulting hydrolysate was suitable for the growth of the strain, but the C/N ratio was not high enough for polymer production. However, the supplementation of the hydrolysate with further glucose and salts increased the ratio and supplied the nutritional requirements of the microorganism, leading to the production of poly(3-hidroxybutyrate). With the data obtained in this first study, the scale-up of the biopolymer production process was carried out on a laboratory scale (5 L). Bearing this in mind, the hydrolysate of the organic fraction of urban solid waste was used as the initial culture medium (batch phase) due to its nutritional balance, and a concentrate of plum residues, rich in sugars, as a "feed" in the fed-batch phase. Finally, the techno-economic viability of the process was evaluated through a simulation with SuperPro Designer® software. The results obtained indicated that, although the production of poly(3-hydroxybutyrate) is technically viable, it is not economically viable, mainly due to the low yield of the fermentation.

Furthermore, following a similar methodology, a strategy to obtain an amino acid concentrate from the overproduction of compost generated with the organic fraction of municipal solid waste was designed. This strategy consisted of protein hydrolysis carried out with a combination of several enzymes. The hydrolysate obtained in this first stage was supplemented with salts and tested as a nitrogen source for the fermentative production of lactic acid by *Lactobacillus fermentum* ATCC 9338 and *Lactobacillus plantarum* NCIMB 8826. The lactic acid concentrations achieved were close to 10 $g \cdot L^{-1}$. Despite the fact that the tests were carried out in a flask and that it is necessary to carry out studies at larger scales, the results obtained are promising and represent a good starting point for developing more sustainable methods of producing lactic acid at a lower economic cost.

The innovation of these approaches was to take advantage of the high generation of waste for the production of high-value compounds, thus contributing to the creation of circular economy scenarios around them.

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LIST OF ABBREVIATIONS

APAC	Asia Pacific
CAGR	Compound Annual Growth Rate
CCR	Carbon Catabolite Repression
DO	Dissolved Oxygen
EC	European Commission
EU	European Union
LAB	Lactic Acid Bacteria
LCA	Life Cycle Assessment
LCL-PHA	Large Chain Length-PHA
MCL-PHA	Medium Chain Length-PHA
MFA	Material Flow Analysis
MSW	Municipal Solid Waste
NPV	Net Present Value
OECD	Organization for Economic Co-operation and Development
OF	Organic Fraction
OFMSW	Organic Fraction of Municipal Solid Waste
P(3HB)	Poly(3-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)

PBAT Polybutylene adipate terephthalate

PBS Polybutyrate succinate

PCL Polycaprolactone

PE Polyethylene

PEA Poly(ester amide)

PET Polyethylene terephthalate

PHA Polyhydroxyalcanoate

PHB Polyhydroxybutyrate

PHBv Polyhydroxybutyrate-co-valerate

PLA Polylactic acid

PMMA Polymethacrylate

PP Polypropylene

PS Polystyrene

PVC Polyvinyl chloride

ROI Return of Investment

ROP Ring Opening Polymerization

SCL-PHA Short Chain Length-PHA

TEM Transmision Electron Microscope

ANTECEDENTES

BACKGROUND

ANTECEDENTES

Un residuo se define como "*la parte o porción de un todo después de quitar otra parte*" (RAE). La palabra proviene del latín "*residuum"* y significa *"aquello que resta, que queda".* El término engloba varios significados, dependiendo del área en la cual se utilice. Así, en el ámbito ecológico o ambiental se refiere a *"cualquier sustancia u objeto del cual su poseedor se desprenda o tenga la intención o la obligación de desprenderse"* es decir, son los materiales que dejan de tener valor tras su utilización (EC, 2008).

Desde el origen del ser humano, su actividad ha generado residuos. Inicialmente, estos residuos procedían principalmente de la alimentación y de la construcción de objetos sencillos, por lo tanto, se integraban fácilmente en la naturaleza sin un impacto significativo. No obstante, con el desarrollo de la sociedad y la tecnología, la generación de residuos se incrementó, convirtiéndose en un problema. Este problema se agudizó a partir de la Revolución Industrial debido al aumento de las actividades industriales y al crecimiento de la población. Pero fue a partir de la segunda mitad del siglo XX, con la expansión de una economía basada en el consumo, cuando los residuos comenzaron a considerarse un problema medioambiental (Scheel, 2016). La creación de nuevos materiales alteró la composición de los residuos producidos, disminuyendo la fracción orgánica y aumentando otras fracciones como la de plástico, la de vidrio y la de papel. Hasta esa época la gestión de los residuos se enfocaba al aprovechamiento agrícola como fertilizante y el resto se eliminaba en vertederos o se incineraba (Agnoletti and Serneri, 2014).

Como respuesta a los problemas causados por la generación de residuos, se han desarrollado sistemas de gestión como, la recogida selectiva, el reciclaje, la valorización energética, el vertido controlado y el compostaje entre otros (Ma et al., 2016). Sin embargo, la fabricación de materiales más resistentes y duraderos, el aumento del consumo, el agotamiento de materias primas y el incremento de la población mundial, que se espera que supere los 9.000 millones en los próximos años, no han hecho más que agudizar el problema. En consecuencia, la sociedad actual se enfrenta al reto de diseñar nuevas estrategias de gestión de los residuos. Para superar

este reto es necesario plantear un cambio de modelo, donde los residuos dejen de ser considerados desechos y puedan volver a incorporarse a las cadenas de valor convertidos en materias primas (Peter and Jakob, 2015).

A este respecto, la Economía Circular se postula como un modelo sostenible en el cual se prioriza mantener en la economía el valor de los productos, los materiales y los recursos durante el mayor tiempo posible, reduciendo al mínimo la generación de residuos. Además, contempla la reutilización y el reciclado de flujos clave de residuos, como los residuos urbanos (Ghisellini et al., 2016; Tisserant et al., 2017). En este contexto, el uso de la fracción orgánica de residuos urbanos como materia prima para la producción de compuestos de interés, como plásticos biodegradables es una alternativa viable y a priori más sostenible que su eliminación (Matsakas et al., 2017). Dicha alternativa permite dar valor a un residuo transformándolo en un recurso, mientras que al mismo tiempo se evita su acumulación en vertederos. Cabe mencionar que esta estrategia no está en disputa con las ya existentes y, por lo tanto, son complementarias.

La transformación de un residuo orgánico en un producto nuevo, implica un cambio en su estructura química. Estos cambios pueden realizarse química o biológicamente, dependiendo del objetivo. Generalmente, las transformaciones químicas requieren el uso de catalizadores para facilitar las reacciones, en cambio, en los procesos biológicos se utilizan los residuos, como nutrientes, en el cultivo de microorganismos (Xiong et al., 2019). Dichos microorganismos, a través de su metabolismo, generan compuestos que tienen una determinada función para ellos o que simplemente son desechos. Muchos de estos compuestos son valorados por la industria y presentan multitud de aplicaciones. Por ejemplo, los polihidroxialcanoatos son producidos por diferentes tipos de bacterias y se utilizan en la fabricación de plásticos biodegradables, el ácido láctico es generado por las bacterias lácticas y es muy demandado como "building block" en la fabricación de otros compuestos y el ácido succínico es sintetizado por *Actinobacillus succinogenes* y se emplea en la industria alimentaria como saborizante (Ismail et al., 2018; Kourmentza et al., 2017; Salvachúa et al., 2016). Sin embargo, antes de utilizar los

residuos en la producción de nuevos compuestos, ya sea por métodos químicos o biológicos, es necesario la aplicación de una serie de pretratamientos (Koutinas et al., 2014; Shrotri et al., 2017). Estos tratamientos previos tienen la función de separar de la matriz los compuestos (azúcares, proteínas, aminoácidos, etc.) que se utilizarán como materias primas en las trasformaciones químicas o biológicas. La forma más común de extraer estos compuestos es mediante tratamientos termo-químicos (ácidos o bases), enzimáticos o la combinación de ambos (Kumar et al., 2019). El estudio y la optimización de los parámetros que afectan a los tratamientos vistos anteriormente es fundamental para que el proceso sea viable, ya que obtener concentraciones de los compuestos de partida afectará positivamente al rendimiento final de la transformación del residuo en un producto.

Por otro lado, estudiar la viabilidad tecno-económica del proceso desarrollado es importante a fin de valorar su rentabilidad. A este respecto, para poder llevar a cabo una evaluación tecno-económica es necesario escalar el proceso, por lo menos a escala laboratorio. El escalado representa una actividad fundamental para transportar el conocimiento generado durante la fase experimental a una realidad.

En este contexto, el presente trabajo propone alternativas, enmarcadas en una bioeconomía circular, para la gestión de los residuos municipales. Estas alternativas se basan en el uso de la fracción orgánica para la producción de compuestos de interés mediante transformaciones biológicas (fermentación aerobia).

INTRODUCTION

INTRODUCTION

INTRODUCCIÓN

En esta sección se realiza una revisión de la información más importante relacionada con esta tesis doctoral, que proporciona el contexto para la posterior interpretación de los resultados experimentales presentados. El propósito es, por un lado, dar una visión general de las causas que están llevando al colapso del modelo económico actual, así como de las alternativas más prometedoras que se están introduciendo, y por el otro, plantear la producción de bioplásticos (poli(3-hidroxibutirato) y ácido poliláctico) a partir de la fracción orgánica de residuo sólido urbano, como un ejemplo aplicable de modelo sostenible.

1. Avanzando hacia un modelo más sostenible

1.1. Economía lineal

Desde principios de la década de los 70, la velocidad de consumo anual de los recursos naturales ha ido en aumento. De hecho, en 2019 la humanidad consumió los recursos 1,75 veces más rápido de lo que los ecosistemas se regeneran ("Global Footprint Network," 2019). Esta tendencia es una consecuencia del modelo económico actual, basado en una economía lineal, que básicamente se puede resumir en "adquirir, usar y desechar" (figura1).

Figura 1: El modelo económico lineal.

Dicho modelo, se basa en la distribución geográfica desigual de los recursos. Históricamente las zonas más desarrolladas (sociedades occidentales) han concentrado una mayor cantidad de recursos materiales y de energía, esta abundancia ha contribuido a que las materias primas convencionales (petróleo,

minerales, fibras, etc.) hayan sido muy baratas (Sariatli, 2017). La consecuencia de disponer de una materia prima abundante y con bajos precios, marca una tendencia a no reciclar, no reutilizar ni gestionar de manera adecuada los residuos, esto junto con la falta de medidas regulatorias, que penalicen estas prácticas, no ha hecho más que favorecer este esquema (Andrews, 2015).

Con el inicio del nuevo siglo se ha advertido un cambio de tendencia, los precios de los materiales que anteriormente disminuían ahora aumentan. Este incremento de precios se puede atribuir en parte a la enorme presión sobre los recursos, que cada vez son menos abundantes y accesibles. Esta nueva tendencia sumada a una feroz competencia, obliga a las empresas a asumir costes en lugar de incrementar los precios de los productos, lo cual se traduce en una disminución de beneficios (Ellen Mcarthur, 2014). Además, varios estudios auguran un agravamiento de estos efectos debido al aumento de la clase media global, que se ha incrementado enormemente en los últimos años (Kharas, 2017; Koo, 2016).

Es incuestionable la contribución de la economía lineal al desarrollo de la sociedad actual, sin embargo, cada vez hay más estudios que advierten de la ineficacia y riesgos de este modelo. Mittal and Gupta. (2015) describen las consecuencias medioambientales, económicas y para la salud, de la sobreexplotación de los recursos no renovables. Otros autores, exponen que el diseño de productos con una vida útil demasiado corta genera un alto consumo de recursos, así como una gran cantidad de residuos (Cooper, 2016), advierten del excesivo consumo de energía que supone un modelo de producción lineal (Michelini et al., 2017) y analizan la relación entre ciertos problemas sociales como la pobreza y la desigualdad con una economía lineal (Galanis et al., 2019). Todos estos factores alertan sobre la insostenibilidad de este sistema y la necesidad de un cambio. Actualmente los modelos que más atención están recibiendo por parte de la comunidad científica e instituciones, son aquellos que se basan en la economía circular y bioeconomía (Geissdoerfer et al., 2017; Murray et al., 2017; Sariatli, 2017).

1.2. Economía circular, bioeconomía y bioeconomía circular

En la última década, la economía circular está experimentando un gran impulso, que en parte se debe a los problemas generados por el modelo lineal y al aumento de la población (Korhonen et al., 2018). El concepto se basa en considerar como nutrientes a todos los materiales involucrados en los procesos industriales y comerciales, de los cuales hay dos categorías principales: i) nutrientes biológicos, diseñados para reingresar a la biosfera de manera segura y construir capital natural (bioeconomía) y ii) nutrientes técnicos, que son de una alta calidad y están diseñados para circular durante mucho tiempo sin entrar en la biosfera. En otras palabras, es una estrategia que implica reutilizar, reparar, renovar y reciclar materiales y productos existentes todas las veces que sea posible para maximizar la retención de valor económico (generando valor añadido) (figura 2).

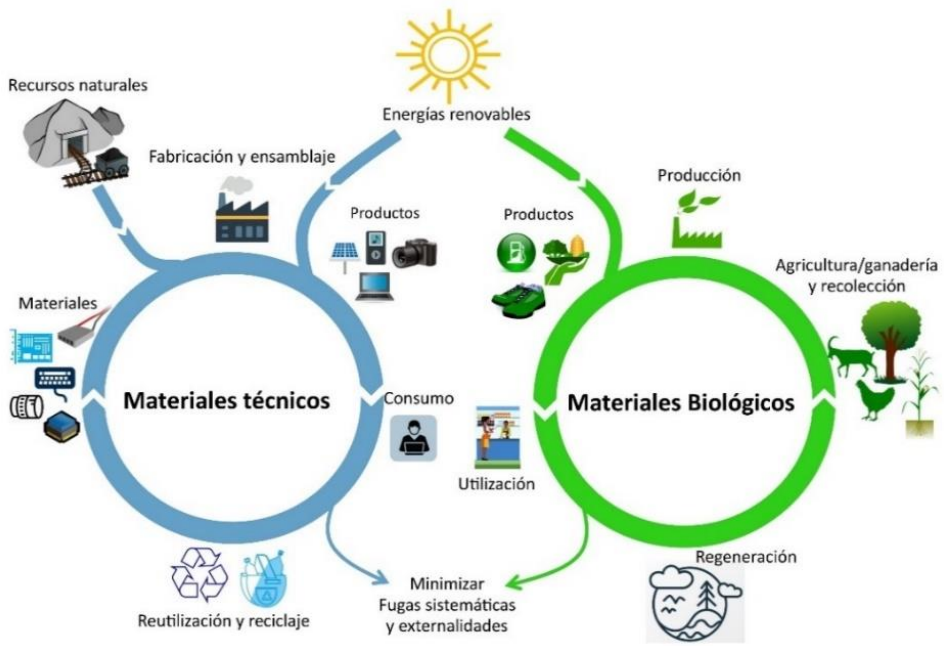

Figura 2: Modelo de economía circular basado en el modelo propuesto por la fundación Ellen McArthur (Ellen Mcarthur, 2014).

De esta forma, el ciclo de vida de los productos se extiende dando como resultado un modelo económico sostenible, que no solo tiene en cuenta la sobreexplotación de

recursos, sino también el consumo de energía, las emisiones, la generación de residuos y aspectos sociales cómo la creación de puestos de trabajo, el aumento de las relaciones entre sectores industriales y la mejora de la salud ciudadana (Skanberg et al., 2014). Los procesos creados son de ciclo cerrado, ayudando a minimizar el desperdicio, mientras que al mismo tiempo se respetan los entornos naturales, sociales y globales.

A pesar de los esfuerzos, existen en la actualidad una serie de barreras que impiden una transición rápida hacia una economía circular, como i) el escaso apoyo y estimulación por parte de los gobiernos a través de financiación, formación y políticas, además de la falta de armonización de los conceptos de economía circular que sirvan como cimientos para que se establezcan estándares certificables válidos, ii) la falta de conciencia ambiental, la desinformación y el desconocimiento por parte de proveedores y clientes son factores que ralentizan el cambio, iii) el coste de la innovación en tecnologías para adaptarse a un modelo circular penaliza mucho a cierto tipo de empresas, lo que dificulta mucho la transición y iv) la falta de competencias tecnológicas, conocimientos y habilidades técnicas, pueden ser un hándicap a la hora diseñar sistemas basados en la reutilización, reacondicionamiento y reciclado, es decir para implementar los principios de una economía circular (Ritzén and Sandström, 2017).

La bioeconomía tiene como premisa utilización de recursos renovables de base biológica para generar productos con valor añadido, como alimentos, bioproductos y bioenergía (EC, 2012), y un desarrollo sostenible a través del uso eficiente de los recursos naturales. El objetivo final es la sustitución de los combustibles fósiles con el fin de mitigar las emisiones de gases de efecto invernadero y disminuir la huella de carbono (Ingrao et al., 2018). Si bien algunas definiciones resaltan más el lado biotecnológico y el lado de los recursos biológicos de la bioeconomía, otras lo ven como una garantía de producción de alimentos, energía, productos y servicios más sostenible y amigable con el medio ambiente (Bugge et al., 2016; Meyer, 2017). En esta Tesis, la bioeconomía se identifica con la innovación en nuevos productos basados en residuos orgánicos.

Si se analiza la contribución de la bioeconomía a la sostenibilidad, priorizando la prosperidad económica, asumiendo el bienestar ambiental y tratando libremente el aspecto social, surgen controversias. Según Pfau et al. (2014), hay situaciones en las que la bioeconomía no puede considerarse sostenible, por ejemplo, en relación a los problemas de uso de la tierra, la reducción ambigua de las emisiones de gases de efecto invernadero y los efectos negativos causados por la producción de biomasa en los sistemas naturales (Meyer, 2017; Priefer et al., 2017).

La bioeconomía y economía circular tienen un objetivo común: una sociedad más sostenible, eficiente en el uso de recursos y con una menor huella de carbono. De la intersección de ambas, se plantea el concepto de "bioeconomía circular" (figura 3), en la que se optimiza la utilización de recursos alargando su vida útil, reduciendo las emisiones de carbono fósil adicionales y reemplazando el carbono fósil por carbono de origen renovable (residuos de agricultura, forestales, marinos, etc.).

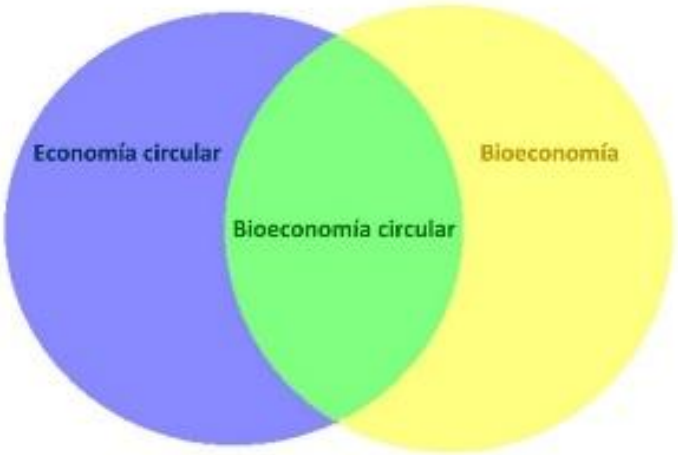

Figura 3: Bioeconomía circular, fruto de la intersección entre economía circular y bioeconomía.

Se trata de dos perspectivas diferentes y complementarias (Aguilar et al., 2018). Ambos conceptos son dependientes el uno del otro dado que las corrientes orgánicas (alimentos, deshechos agroalimentarios, biocombustibles y deshechos de procesos orgánicos) únicamente lograrán integrarse en la economía circular mediante

procesos de bioeconomía, a la vez que la bioeconomía se verá favorecida enormemente por una mayor circularidad (Carus and Dammer, 2018)

La transición hacia una bioeconomía circular conlleva la búsqueda de métodos específicos que evalúen su progreso. Para ello, se requieren herramientas que sirvan de apoyo en la toma de decisiones y permitan formular políticas correctas relacionadas con la economía circular y la bioeconomía (Moraga et al., 2019). En los últimos años se han desarrollado una amplia gama de indicadores de circularidad basados en métodos de evaluación conocidos: energía, análisis de flujo de materiales (MFA, Material Flow Analysis), análisis de ciclo de vida (LCA, Life Cycle Assessment), emisiones de CO_2 y retornos económicos.

En esta línea, académicos, empresarios y políticos coinciden en la necesidad de crear métodos robustos que permitan medir de una manera sencilla y precisa la circularidad de los procesos y de los productos, con el fin de aumentar la eficiencia del modelo (Saidani et al., 2019).

1.3. Economía circular y bioeconomía en la Unión Europea y en España

Desde hace tiempo, la necesidad de hallar fuentes alternativas de recursos ha llevado a la Unión Europea (EU, European Union) a pensar nuevas estrategias basadas en la innovación, que durante 30 años se han ido plasmando en diferentes planes y acciones (Figura 4). Esto ha permitido a Europa, a través de sus políticas basadas en la bioeconomía y economía circular, colocarse a la cabeza mundial en materia de sostenibilidad. En los últimos años, destaca la estrategia de bioeconomía (EC, 2012), que afirma que Europa necesita cambiar radicalmente su enfoque de producción (consumo, procesamiento, almacenamiento, reciclaje y eliminación de recursos biológicos), para hacer frente a una población mundial en aumento, al agotamiento de los recursos, al aumento de las presiones ambientales y al cambio climático (Hess et al., 2016).

La actualización de 2018 hizo hincapié en acelerar el despliegue de una bioeconomía europea sostenible para maximizar su contribución a la Agenda 2030, así como para cumplir los objetivos alcanzados en el al Acuerdo de París (EC, 2018).

En 2020 la Comisión Europea (EC, European Commission) ha adoptado un nuevo plan de acción de Economía Circular, que se asienta sobre el anterior Plan presentado en el año 2015 (EC, 2020; EC, 2015). Este nuevo plan, forma parte de Pacto Verde Europeo y se centra en el diseño y la producción de una economía circular, con el objetivo de garantizar que los recursos se mantengan en la economía el mayor tiempo posible (EC, 2019).

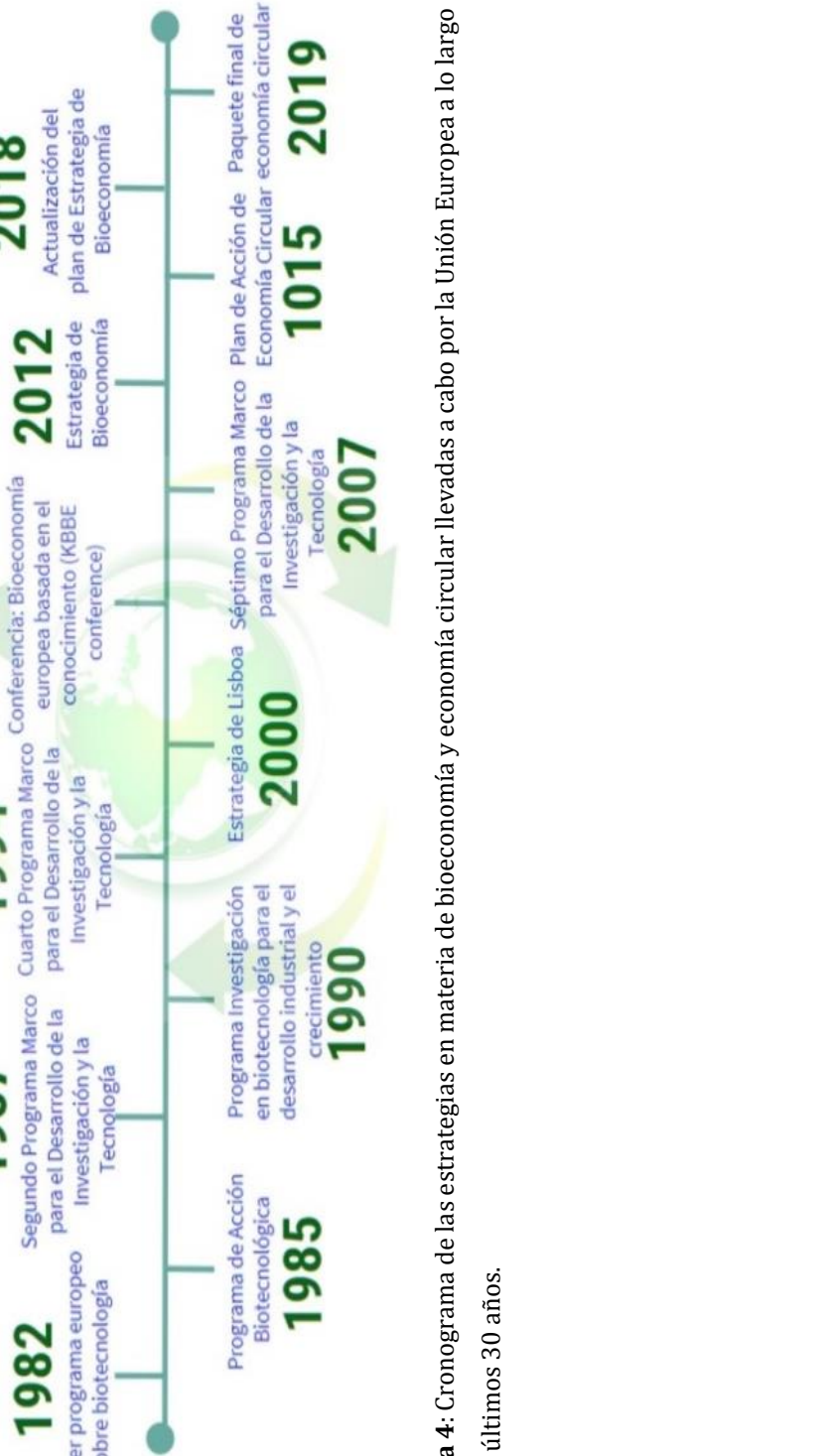

Figura 4: Cronograma de las estrategias en materia de bioeconomía y economía circular llevadas a cabo por la Unión Europea a lo largo de los últimos 30 años.

Siguiendo la línea trazada por Europa en cuestión de bioeconomía y economía circular, España creó un grupo de trabajo en 2013 cuyo objetivo principal era evaluar la oportunidad de desarrollar una estrategia de bioeconomía. El grupo analizó y utilizó como referencia las estrategias publicadas por la Organización para la Cooperación Económica y el Desarrollo (OECD, Organization for Economic Co-operation and Development), la UE y varios países entre los que se encontraban Alemania, EEUU, Irlanda y Países Bajos. Se tomó en cuenta la fortaleza de los sectores económicos detrás del concepto de bioeconomía, la participación de la sociedad y los desafíos planteados en el "*Plan Nacional de Investigación e Innovación Científica y Tecnológica*" (2013-2016) (MINECO, 2013). La conclusión general fue que la bioeconomía merece una estrategia concreta en España, teniendo en cuenta sus propias características; lo que propició el lanzamiento en 2016 de la Estrategia Española de Bioeconomía–Horizonte 2030 (Lainez et al., 2018; MINECO, 2015). Además, con el fin de cumplir con el objetivo de involucrar a los principales agentes económicos y sociales de España en la transición hacia un nuevo modelo económico, el Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente publicó el "*Pacto por una Economía Circular*", firmado por más de 300 instituciones, y paralelamente se aprobó. la "*Ley de Cambio Climático y Transición Energética*" que supuso un apoyo esencial para el desarrollo de la Economía Circular a nivel estatal y autonómico (COTEC, 2019).

A nivel regional, el País Vasco en 2019 presentó "*La nueva Estrategia de Economía Circular de Euskadi 2030*", que plantea aumentar en un 30 % la productividad material, duplicar la tasa de uso de material circular, y reducir en un 40 % la tasa de generación de residuos (COTEC, 2019; EUS, 2019). Esta estrategia se coordina con otras acciones y líneas de desarrollo, como el "*Plan de prevención y gestión de residuos 2020*" (EUS, 2015), el "Plan de Ciencia, Tecnología e Innovación Euskadi 2020" (EUS, 2014a), "*Programa marco ambiental 2020*" (EUS, 2014b) y la "*Red Circular Basque*" (Circular Basque).

2. Bioeconomía circular aplicada a la producción de plásticos biodegradables

2.1. Plásticos convencionales y bioplásticos

La invención del plástico es un hito importante que ha conducido a una mejora de la calidad de vida del ser humano. Desde su primera síntesis a principios del siglo XX, los plásticos han ido sustituyendo, en la fabricación de productos de consumo, a muchos tipos de materiales como madera, metales, cerámicas y fibras naturales. Esta tendencia se debe en buena parte a que es un material barato, y además tiene unas propiedades que lo hacen extremadamente versátil (Wong et al., 2015). Como consecuencia, su utilización ha ido creciendo paulatinamente y desde el año 2014, que se superó la barrera de los 300 millones de toneladas, la producción anual no ha hecho más que aumentar. De continuar con esta tendencia, en el año 2050, la producción mundial podría alcanzar los 1600 millones de toneladas (figura 5) (Geyer et al., 2017; Lebreton et al., 2017).

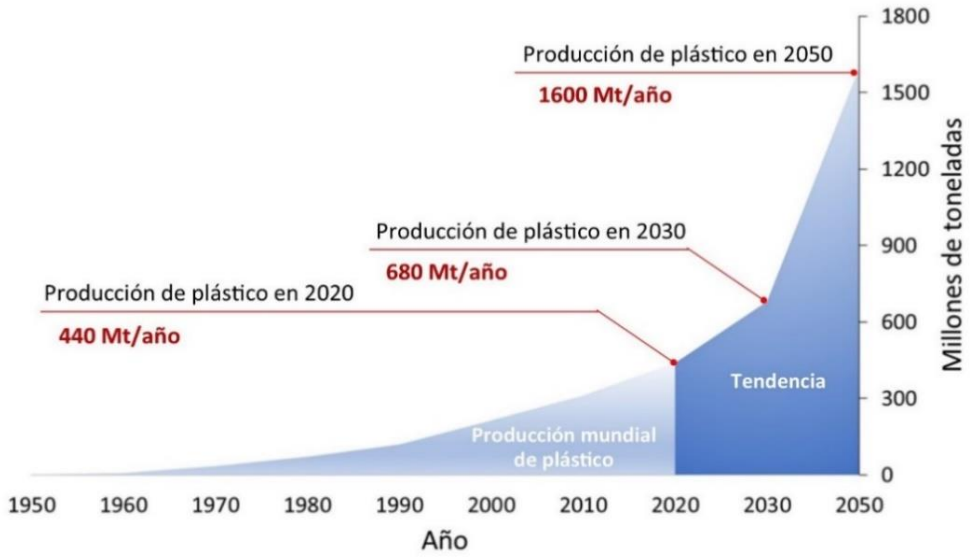

Figura 5: Producción mundial de plástico en millones de toneladas. El área en azul claro muestra los datos históricos de 1950 a 2020 y el área azul oscuro la tendencia hasta 2050. Fuente: (Bergmann et al., 2015; Plastics Europe, 2015; Geyer et al., 2017), Maphoto/Riccardo Pravettoni.

La variedad de los materiales plásticos es muy grande, aunque, un porcentaje muy alto de los usados cotidianamente (90 %) corresponde a los seis más utilizados: i) polietileno (PE, Polyethylene), ii) polipropileno (PP, Polypropylene), iii) policloruro de vinilo (PVC, polyvinyl chloride), iv) poliestireno (PS, polystyrene), v) politereftalato de etileno (PET, Polyethylene terephthalate) y iv) polimetilmetacrilato (PMMA, polymethacrylate). El resto corresponde a otros plásticos de ingeniería, donde destacan las poliamidas y el poliuretano. Del grueso de producción, la mayor parte es destinado a los sectores del embalaje y de la construcción (Ray and Cooney, 2018).

A pesar de que el plástico es un material extraordinario, hay ciertos factores que a día de hoy lo hacen insostenible. Por un lado, el modelo actual de producción, basado en una economía lineal, demanda grandes cantidades de recursos naturales, como petróleo y gas natural, para su obtención. Tras su utilización, solamente una pequeña parte es reciclada, el resto es eliminado en vertederos, en el medio natural o directamente incinerado (Geyer et al., 2017; Leal Filho et al., 2019). Además, los recursos fósiles destinados a su fabricación son limitados, su precio es volátil y compiten con otros sectores importantes como la movilidad y la generación de energía (Storz and Vorlop, 2013). Por otro lado, una de sus propiedades más reseñables, la estabilidad química, es al mismo tiempo una gran desventaja ya que para su descomposición completa son necesarios varios siglos. Estos factores, sumados al hecho de que casi la mitad de los plásticos producidos tienen una vida estimada de 1 mes (en el ciclo de consumo), están generando graves problemas de salud y ambientales, sobre todo en los medios acuáticos. (Hahladakis and Iacovidou, 2018).

Gran parte de los residuos plásticos llegan a los océanos, a través de las fuentes de agua dulce (ríos) o directamente desde zonas costeras, y se acumulan en diferentes puntos como los fondos, las costas o en la superficie, formando grandes cúmulos (Lebreton et al., 2017). Su naturaleza (baja densidad, persistencia, forma, etc.) facilita su dispersión por los ecosistemas marinos, causando un gran impacto en la fauna y flora; además, la erosión causada por los embates fragmenta estos

materiales en partículas muy pequeñas (microplásticos) que se dispersan por todo el océano, y cuyos efectos a aún son desconocidos. La concentración de macro y microplásticos en los mares es proporcional a los insumos de residuos, esto unido a su pequeño tamaño hace que su eliminación sea extremadamente difícil, siendo la reducción de su producción la estrategia más efectiva (Jambeck et al., 2015); sin embargo, para las próximas décadas se prevé un aumento de la producción. Esto implica que para disminuir los residuos son necesarias estrategias basadas en una economía circular donde la vida de los materiales debe extenderse o bien incrementarse el porcentaje de material reciclado. A pesar de los esfuerzos, a nivel mundial únicamente se recicla entre el 14 y el 18 %, pero incluso los países más concienciados no llegan a niveles demasiado altos de reciclado (OECD, 2018).

Los mayores obstáculos son la falta de disponibilidad de puntos de recolección, la contaminación de la materia prima de reciclaje (residuos orgánicos, compuestos químicos, metales, etc.), la limitada comerciabilidad del material reciclado y la enorme diversidad de polímeros existente. Concretamente este último obstáculo es el más complicado de solventar porque las diversas estructuras y propiedades moleculares de cada tipo de plástico, es lo que les confieren comportamientos de fusión, reológicos y de estabilidad térmica diferentes, que finalmente, termina por afectar a las características del material reciclado resultante (Hahladakis and Iacovidou, 2018; Singh et al., 2017). Esto es importante ya que los compuestos reciclados tienen unas características muy diferentes a las de los materiales de partida y por ello no pueden sustituirlos en muchos casos. Por ejemplo, no está permitido el uso de materiales reciclados para fabricar envases destinados a la alimentación ya que podrían tener restos de plásticos tóxicos (PVC), y tampoco se pueden utilizar en construcción de piezas para maquinaria porque podría afectar a la seguridad (Grigore, 2017; Pivnenko et al., 2016). Afortunadamente, hay otros ámbitos donde su uso está autorizado como en el mobiliario urbano, en dispositivos electrónicos, en algunas prendas de vestir, en carreteras, etc. (Dalen et al., 2017; Jalaluddin, 2017; Rane et al., 2019).

Una de las alternativas basadas en una bioeconomía circular, es la sustitución de los plásticos convencionales por plásticos procedentes de fuentes renovables, como plantas, microorganismos, restos de animales y todo tipo de residuos orgánicos. Estos tipos de plásticos alternativos, y fabricados a partir de fuentes renovables, se les denomina comúnmente bioplásticos, y para ser unos buenos sustitutos de los materiales convencionales deben tener características similares (Karan et al., 2019).

El término bioplástico fue acuñado por la Asociación Europea de Bioplásticos (European Bioplastics e.V.), quien los definió como una familia de materiales de base biológica, biodegradables o con ambas propiedades. Con bioplásticos de base biológica se refiere a que están fabricados a partir de biomasa en lugar de fuentes no renovables, mientras que con biodegradables a que pueden degradarse por la acción de los microorganismos presentes en el medio natural. La biodegradabilidad no depende del tipo de materia prima, sino que está vinculada a la estructura química del compuesto, además llevar la etiqueta de bioplástico no siempre implica ser biodegradable. En la figura 6 se detalla la clasificación de los bioplásticos disponibles según su origen (renovable o no renovable) y biodegradabilidad.

Muchos de los plásticos más comunes, como el polietileno, el polipropileno y el cloruro de polivinilo, también pueden fabricarse con recursos renovables, por ejemplo, con bioetanol. Estas poliolefinas, no biodegradables y de base biológica, tienen estructuras químicas idénticas a las de los productos procedentes de fuentes no renovables; por lo tanto, no hay diferencia en las consecuencias al final de la vida útil de ambas (Sidek et al., 2019). No obstante, existen dos diferencias principales entre los bioplásticos y los plásticos comunes, los costes de producción y la huella ambiental. La producción de plásticos convencionales es menos costosa debido a que la industria encargada de su fabricación tiene un mayor recorrido y, además, su capacidad de producción es mucho más amplia. Por otro lado, durante la incineración de los bioplásticos no se libera a la atmósfera CO_2 adicional, ya que el CO_2 liberado fue inicialmente adquirido por la biomasa utilizada en su síntesis (Ross et al., 2017).

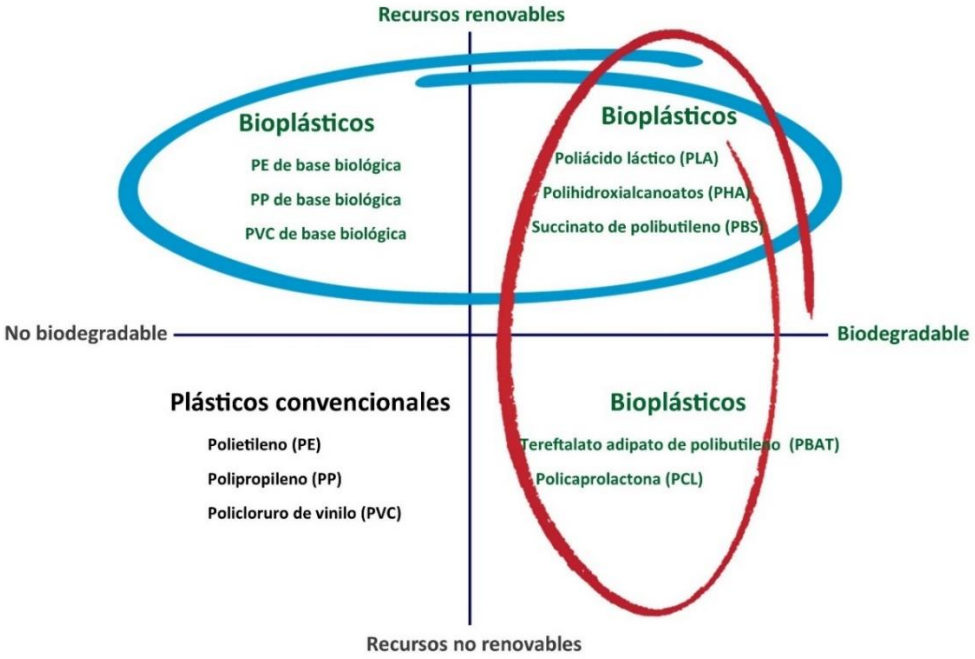

Figura 6: Clasificación de los plásticos por origen y biodegradabilidad. Fuente: "European Bioplastics," 2019.

Todos estos plásticos de origen biológico, pero no biodegradables, son conocidos como "duraderos", y se utilizan normalmente en aplicaciones donde es necesaria una alta durabilidad de los materiales, como por ejemplo en el campo de la aeronáutica, el automovilismo o la industria textil, etc., (Aeschelmann and Carus, 2015; Andreeßen and Steinbüchel, 2019; Zhao et al., 2020). De forma complementaria, se están desarrollando nuevos y mejores bioplásticos con alto potencial de degradación (plásticos biodegradables de base biológica). Su interés creciente está relacionado en gran parte con la preocupación generada por la enorme producción de residuos plásticos (mayoritariamente de un solo uso). Su utilización está enfocada hacia áreas en las que la durabilidad del material no es un requisito importante, siendo sus aplicaciones más interesantes la agricultura, la medicina y el envasado (Ezgi Bezirhan Arikan and Havva Duygu Ozsoy, 2015; Jabeen et al., 2015; Piergiovanni and Limbo, 2016).

Los beneficios que aportan los bioplásticos a la economía y al medio ambiente son amplios. El uso de recursos renovables durante su fabricación refuerza la sostenibilidad, aumentando la eficiencia de los recursos, reduciendo la huella de carbono y disminuyendo el uso de combustibles fósiles. Esto se alinea con los principios de una bioeconomía circular, en la que los plásticos nunca se convierten en residuos, sino que, en su lugar, vuelven a entrar en la economía como nutrientes técnicos o biológicos valiosos (Karan et al., 2019). Como consecuencia, la industria de los bioplásticos ha experimentado un crecimiento, y se prevé que la capacidad de producción mundial de bioplásticos crecerá de 2.12 a 2.42 millones de toneladas de 2019 a 2024 (Figura 7) ("European Bioplastics," 2019).

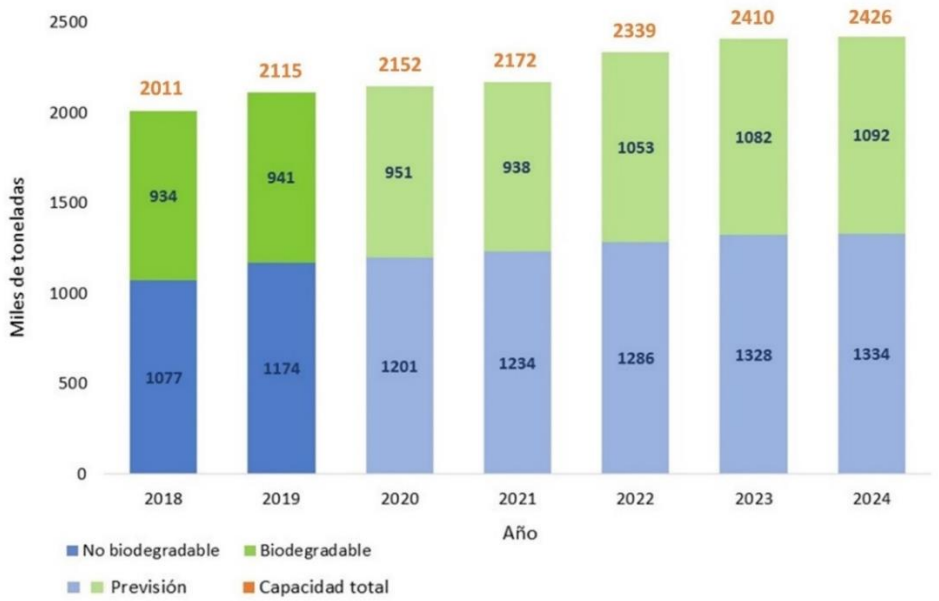

Figura 7: Capacidades de producción global de bioplásticos 2018-2024. Datos derivados de "European Bioplastics".

2.2. Plásticos biodegradables

Los plásticos biodegradables pueden definirse de distintas maneras, desde un punto de vista ambiental son aquellos que se descomponen, principalmente a través de actividades enzimáticas de microorganismos, en CO_2, metano, compuestos inorgánicos o biomasa, dentro de un periodo de tiempo específico (Mohanty et al.,

2002), y desde un punto de vista médico son aquellos plásticos que se descompone debido a la degradación macromolecular con dispersión *in vivo* sin que haya prueba de su eliminación del cuerpo (Bosworth and Downes, 2010). Ambas definiciones son útiles y dependiendo del tipo de aplicación que se dé al compuesto es más práctico utilizar una u otra.

La degradación se produce cuando diferentes factores (luz, calor, cizallamiento, acción de microorganismos, oxidación, etc.) deterioran la estructura del material y los fragmentos resultantes no logran mantener las propiedades mecánicas iniciales. Como consecuencia, el material se vuelve frágil y su vida se ve limitada. Este rasgo posibilita que estos compuestos puedan integrarse en una bioeconomía circular ya que en lugar de acabar como residuos al final de su ciclo de vida (corto en el caso de los plásticos de un solo uso) terminan siendo nuevos nutrientes en la cadena de valor (fertilizantes, fuente de carbono en fermentaciones, etc.) (Kaur et al., 2018). No obstante, el gran desafío está en el hecho de que dichos bioplásticos deben ser estables durante su almacenamiento o uso, momento en el cual ninguna de sus propiedades deber verse alterada, y tras su vida útil deben degradarse en un tiempo razonable. En este sentido, se está intentando adaptar la escala de tiempo, para que la degradación del producto se ajuste a la finalidad (Laycock et al., 2017).

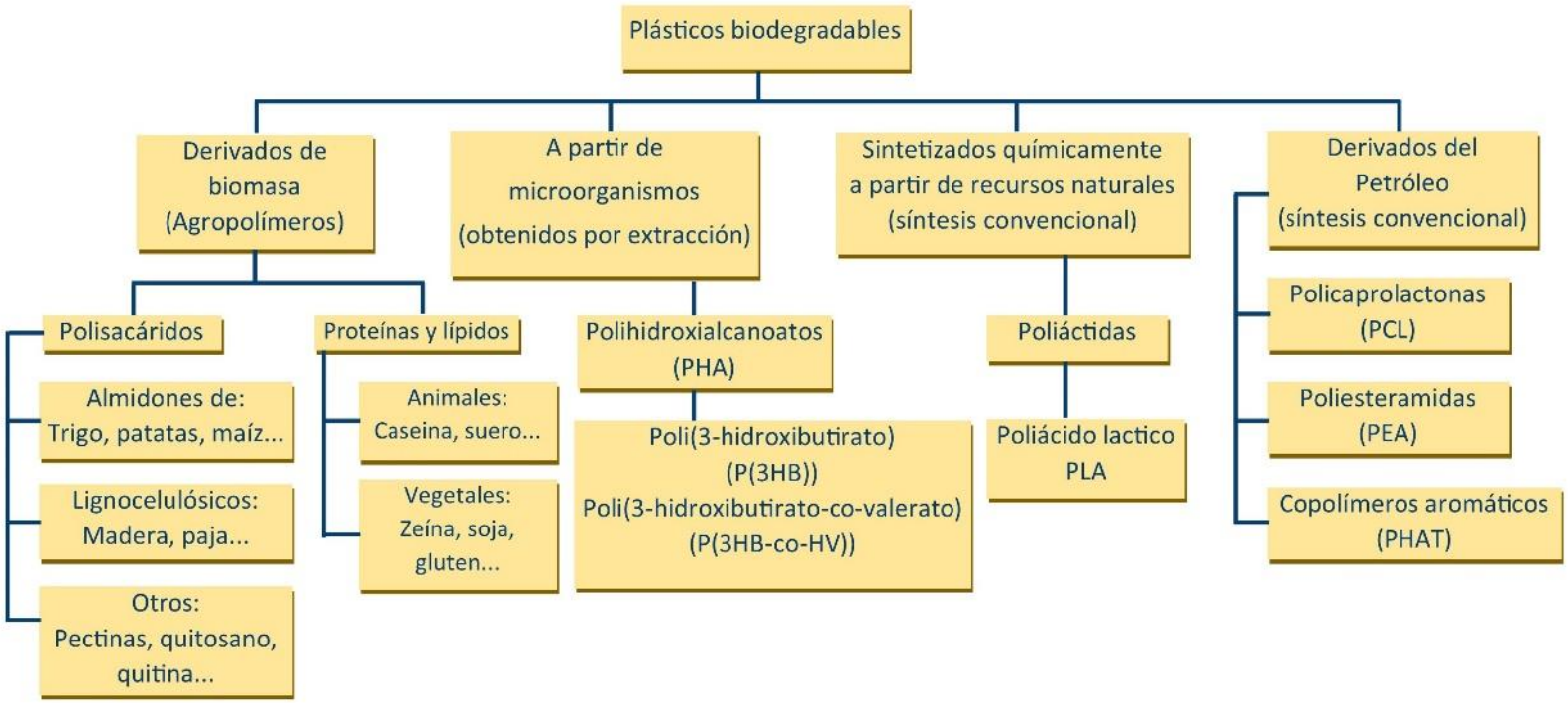

Figura 8: Clasificación de los principales plásticos biodegradables. Fuente: Averous and Boquillon, 2004.

En la actualidad existe una gran diversidad de plásticos o polímeros biodegradables que se pueden clasificar en función de sus propiedades, origen o metodología de síntesis. Numerosos autores han sugerido clasificar los polímeros biodegradables en cuatro categorías basadas en su síntesis (figura 8): (Averous and Boquillon, 2004; Bordes et al., 2009; Sudesh and Iwata, 2008), i) polímeros derivados de recursos agrícolas como polisacáridos (almidón, quitina, etc.), lignocelulosas (madera, paja, etc.), proteínas (colágeno, caseina, etc.), pectinas y lípidos, ii) polímeros obtenidos por producción microbiana, por ejemplo, polihidroxialcanoatos (PHA, polyhydroxyalcanoate) tales como polihidroxibutirato (PHB, polyhydroxybutyrate) y polihidroxibutirato-co-hidroxivalerato (PHBv, polyhydroxybutyrate-co-valetate), iii) polímeros sintetizados químicamente usando monómeros obtenidos de recursos renovables, por ejemplo, ácido poliláctico (PLA, polylactic acid) y iv) polímeros con monómeros obtenidos a partir de recursos fósiles por ejemplo, policaprolactona (PCL, polycaprolactone), poliéster amidas (PEA, poly(ester amide)) y co-poliésteres alifáticos y aromáticos (PBAT, Polybutylene adipate terephthalate).

Los polímeros de las tres primeras categorías son de base biológica, mientras que los de la cuarta, a pesar de ser biodegradables, no tienen un origen renovable. El grupo de plásticos biodegradables con base biológica más empleado son las mezclas de almidón (categoría 1), no obstante, poliésteres como el PLA (categoría 3) y los PHA (categoría 2) están siendo ampliamente investigados ya que sus propiedades, similares a las de los plásticos convencionales, permiten su utilización en innumerables campos y sectores ("European Bioplastics," 2019; Zhao et al., 2020).

En los próximos años se espera que la demanda de este tipo de materiales aumente, debido a la conciencia adquirida por la sociedad respecto a los plásticos convencionales y a la implantación de nuevos modelos de economía circular. Es por ello que muchas investigaciones se están centrando en el desarrollo de nuevos métodos que logren una mejora de las propiedades y un aumento de la eficiencia de producción (Ciriminna and Pagliaro, 2020). Concretamente en este trabajo, se ha

puesto el foco en la producción de ácido láctico (materia prima para la síntesis de PLA) y PHA utilizando residuos orgánicos.

2.2.1. Ácido poliláctico (PLA)

El PLA es un poliéster alifático derivado del ácido láctico conocido desde hace bastante tiempo. En la actualidad su interés se ha visto incrementado debido en buena parte a la demanda de plásticos biodegradables y también al aumento de aplicaciones por el desarrollo de nuevos composites con matrices de PLA (Kaseem, 2019).

Su unidad monomérica, el ácido láctico (ácido 2-hidroxipropanoico), es el ácido hidroxicarboxílico más extendido en la naturaleza, de hecho, es el intermedio metabólico principal en la mayoría de organismos vivos (Ramis-Ramos, 2003). Este ácido orgánico natural se puede producir por síntesis química o por fermentación. La síntesis química del ácido láctico siempre da como resultado la mezcla racémica de los dos isómeros ópticos *D*-(-) y *L*-(+), mientras que la síntesis biológica puede formar uno de los isómeros o la mezcla, dependiendo de las condiciones de cultivo, el tipo de microorganismo y el sustrato utilizado. La pureza óptica del ácido láctico es crucial durante la producción de PLA, ya que pequeñas impurezas enantioméricas pueden cambian drásticamente algunas propiedades del polímero, como la cristalinidad o la tasa de biodegradación (Ahmed and Varshney, 2011).

Las bacterias del ácido láctico (LAB, lactic acid bacteria) y algunos hongos filamentosos son las principales fuentes microbianas de ácido láctico. Los organismos que producen predominantemente el isómero *L* son *Lactobacillus amylophilus*, *Lactobacillus bavaricus*, *Lactobacillus casei*, *Lactobacillus maltaromicus* y *Lactobacillus salivarius*. Otras cepas tales como *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus plantarum* y *Lactobacillus acidophilus* producen el isómero *D* o la mezcla de ambos (Madhavan Nampoothiri et al., 2010).

La síntesis del PLA se puede realizar mediante una reacción de policondensación a partir del ácido láctico. No obstante, esta reacción está muy limitada y da como

resultado polímeros de bajo peso molecular (figura 9a). La causa es el agua generada durante la reacción, que desplaza el equilibrio hacia la despolimerización. Además, esta reacción requiere de largos tiempos y altas temperaturas para que tenga lugar de manera eficiente. Para superar esta limitación se han desarrollado diferentes estrategias. Una de las más utilizadas es la polimerización por apertura de anillo (ROP, ring opening polymerization), que consta de tres pasos. En la etapa inicial se oligomeriza el ácido láctico, a continuación, se dimeriza catalíticamente para producir un monómero de lactida cíclica y finalmente se polimeriza mediante la apertura del anillo (figura 9b) (Ahmed and Varshney, 2011; VanWouwe et al., 2016).

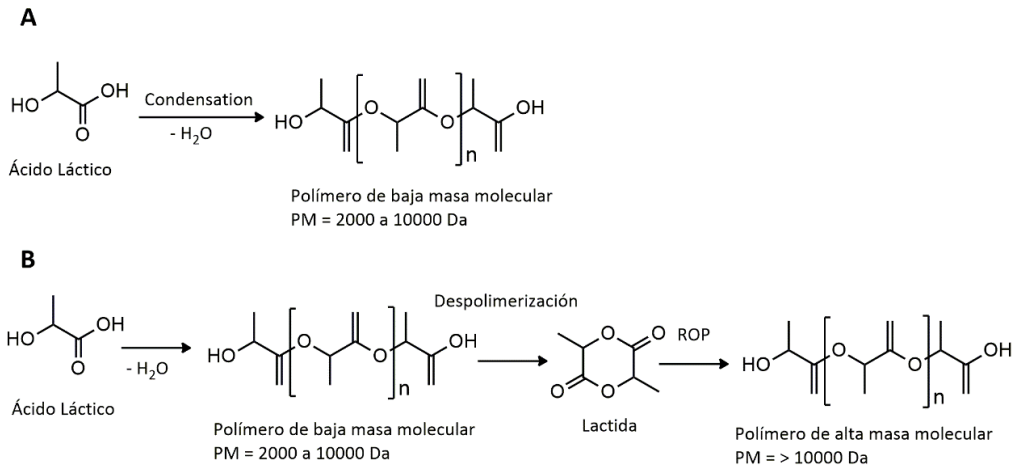

Figura 9: Descripción general de la síntesis de PLA a partir de ácido láctico. A) reacción de policondensación directa, B) reacción de polimerización por apertura de anillo.

La etapa de polimerización no produce agua, hecho que permite la formación de polímeros con altos pesos moleculares. El tipo de isómero de ácido láctico determina la estereoquímica del monómero de lactida, y por lo tanto también la del polímero ya que los enlaces a los carbonos quirales no se rompen durante la polimerización. Como se ha indicado anteriormente este es un aspecto muy importante porque afecta a las propiedades del material.

Las propiedades de PLA dependen principalmente de su composición isomérica, de la temperatura del proceso de síntesis, del tiempo de reacción y de su peso molecular. Cuando el polímero está compuesto de un único isómero (L ó D), su

estructura es semicristalina, tiene un alto grado de cristalinidad, una temperatura de transición vítrea entre 50-80 °C y una temperatura de fusión entre 173-178 °C (Lasprilla et al., 2012). La capacidad de cristalización del PLA se reduce a medida que disminuye la pureza óptica, de hecho, por debajo del 43 % de pureza su estructura es completamente amorfa (Le Borgne et al., 1998).

Los polímeros ópticamente puros son muy resistentes, pero poco tenaces, lo que los hace excesivamente frágiles. No obstante, mediante la copolimerización, la mezcla con otros polímeros o el uso de aditivos, es posible corregir esta fragilidad. También la modificación de algunos factores (parámetros) como la pureza óptica, la temperatura y el tiempo de reacción pueden dar como resultados materiales "a la carta", es decir se pueden manipular las propiedades para satisfacer diferentes necesidades (Farah et al., 2016). Ciertas propiedades mecánicas de este poliéster son equivalentes a las de algunos plásticos convencionales como el PET o el PS (Anderson et al., 2008).

Por otro lado, su comportamiento térmico es el mismo que el de los conocidos como termoplásticos. Esto es, a temperaturas relativamente altas se deforma y derrite y al enfriarse se endurece sin que sus propiedades se vean afectadas, lo que permite que se puedan procesar como la mayoría de termoplásticos.

Aplicaciones del PLA

El sector de los plásticos biodegradables se ha visto favorecido en parte por la concienciación ciudadana sobre los plásticos convencionales, por el cambio hacia modelos más sostenibles y en gran medida por el estudio y mejora de las propiedades de los materiales. En este sentido, el PLA, que actualmente representa un gran porcentaje de los plásticos biodegradables (25 %), se postula como una alternativa esperanzadora a los plásticos no biodegradables ("European Bioplastics," 2019). Sus propiedades, su biodegradabilidad y su biocompatibilidad permiten que este biopolímero pueda ser utilizado en numerosos sectores.

Los principales campos de aplicación de los PLA son: i) la medicina, donde debido a su biocompatibilidad se usa en dispositivos de fijación (fracturas), suturas y sistemas de suministro (Lasprilla et al., 2012); ii) la industria textil, donde se utiliza como material compuesto para generar fibras y fabricar tejidos (puede combinarse con algodón, lyocell o lana) (Avinc and Khoddami, 2009); y iii) el embalaje, principalmente para envolver y conservar alimentos (aplicaciones de un solo uso) (Avérous, 2008).

No obstante, el desarrollo de nuevos materiales basados en el PLA y la mejora de los ya existentes han ampliado el campo de posibles aplicaciones. Algunos ejemplos son la impresión 3D (filamentos), la prevención de incendios (retardantes de llamas), la aeronáutica (fuselaje de los aviones de última generación), la filtración (membranas), etc. (El Magri et al., 2019; Liu et al., 2018; Song et al., 2018; Tiersch and Monroe, 2016).

2.2.2. Polihidroxialcanoatos (PHAs)

Los polihidroxialcanoatos se conocen desde hace décadas, Maurice Lemoigne, en 1920 estudió y caracterizó el poli(3-hidroxibutirato) (P(3HB), poly(3-hydroxybutyrate), uno de los muchos tipos de polímero que comprenden la familia de los PHAs (Ciardelli et al., 2019). Durante muchos años estos materiales, no fueron muy conocidos y no fue hasta década de los 70, durante la crisis del petróleo, cuando recibieron más atención. La escasez de esta materia prima, junto con el aumento de la demanda de los plásticos convencionales llevó a los científicos a centrar la atención en estos compuestos de origen natural. El petróleo es un recurso no renovable y su producción tarde o temprano disminuirá. Afortunadamente, el estudio y la investigación sobre los PHAs ha continuado durante los últimos años (Sudesh and Iwata, 2008).

Los PHAs están compuestos por monómeros de ácido hidroxialcanoico unidos entre sí, formando poliésteres lineales. Su producción está asociada al crecimiento en algunas especies microbianas, mientras que en muchas otras ocurre en condiciones de crecimiento inducido bajo un exceso de carbono y una limitación

simultánea de otros nutrientes como nitrógeno, fósforo, azufre, magnesio u oxígeno. Tal y como se muestra en la figura 10a, el PHA se acumula en el citoplasma en forma de gránulos. Se trata de un compuesto diseñado para almacenar carbono y energía intracelular, aunque parece que también está implicado en otras funciones, como tolerancia al estrés, formación de biofilms y en el mantenimiento del estado redox (Alves et al., 2017; Jiang et al., 2015; Slaninova et al., 2018). Habitualmente en procesos biotecnológicos diseñados para producir PHA se inducen limitaciones de nitrógeno o fósforo ya que se ha visto que el rendimiento de acumulación es mayor (Tu et al., 2019).

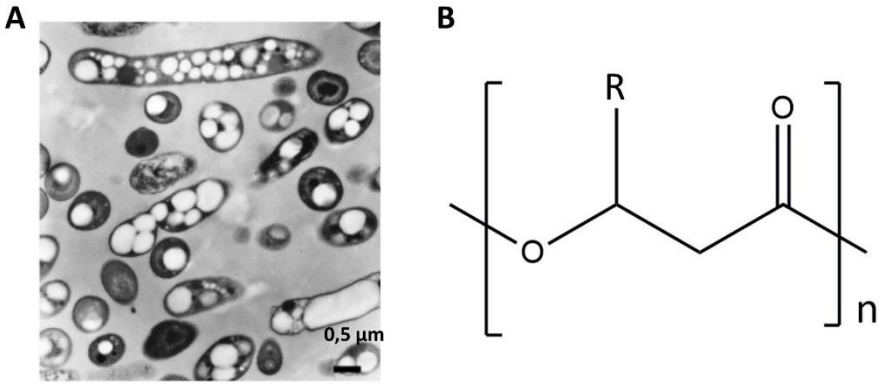

Figura 10: A) Imagen de microscopio electrónico de transmisión (TEM, transmision electron microscope) de los granulos de P(3HB) acumulados en una bacteria. Fuente: (Tian et al., 2005). B) Estructura química general de los PHAs.

La estructura general de los PHAs se muestra en la figura 10b. Cada monómero consta de una cadena lateral (R) que se encuentra unida al carbono 3 o β y cuya longitud puede ir desde 1 átomo de carbono (C1) hasta 13 (C13). La longitud de la cadena lateral y su grupo funcional influye en gran medida en las propiedades físicas de los polímeros, como el punto de fusión, la temperatura de transición vítrea y la cristalinidad, que a su vez determina su aplicación final. También el peso molecular (50-1000 kDa) y la composición pueden variar según el modo y las condiciones de cultivo microbiano, el microorganismo y el proceso de extracción (Mathuriya and Yakhmi, 2017).

A través del metabolismo bacteriano, el sustrato de carbono se transforma en un tioéster de hidroxiacil-CoA. A continuación, la enzima PHB polimerasa (ó PHB sintasa) cataliza la unión entre el grupo carboxilato de un monómero con el grupo hidroxilo del monómero vecino. La intervención de la enzima durante la síntesis asegura que la configuración de los isómeros sea la misma (R (-)), esto garantiza la incorporación esteroespecífica del monómero, que es esencial para la biodegradabilidad y biocompatibilidad del polímero (Philip et al., 2007).

Los PHAs se clasifican en tres grupos diferentes dependiendo del número de átomos de carbono que tengan sus unidades monoméricas: i) PHA de cadena corta (short chain length-PHA ó scl-PHA), que consisten en cadenas formadas por monómeros que tienen de 3 a 5 unidades de carbono, ii) PHA de cadena media (médium chain length-PHA ó mcl-PHA), que constan de cadenas formadas por monómeros con una longitud que va desde 6 a 14 unidades de carbono y iii) PHA de cadena larga (large chain length-PHA ó lcl-PHA), que son polímeros formados por monómeros que tienen más de 14 unidades de carbono (Lu et al., 2009). En la tabla 1 se comparan algunas propiedades de los PHAs con las del polipropileno y poliestireno.

La mayoría de los scl-PHA se caracterizan por ser polímeros bastante frágiles y rígidos, como resultado de su relativamente alta cristalinidad. Presentan altos puntos de fusión y temperaturas de transición vítrea entre 5 y 10 °C (Luef et al., 2015). Tienen propiedades muy similares a las del polímero sintético polipropileno, por lo que en varias ocasiones se han propuesto como posibles sustitutos (Możejko-Ciesielska and Kiewisz, 2016). El homopolímero P(3HB) es el tipo más común de PHA de cadena corta y es el que acapara la mayor producción. No obstante, otros scl-PHA como el poli(4-hidroxibutirato) (P(4HB), poly(4-hydroxybutyrate) tienen una estructura más flexible y resistente. Esta diferencia es debida principalmente a la longitud de las cadenas laterales (Olivera et al., 2010). También existen copolímeros como el poli(3-hidroxibutirato-co-3-hidroxivalerato) (P(3HB-co-3HV)) que son menos frágiles y rígidos, a la vez que mantienen algunas de las propiedades mecánicas asociadas al P(3HB) (Anjum et al., 2016).

Tabla 1: Comparación de algunas propiedades físicas y mecánicas de los tres grupos de PHA (scl, mcl y lcl) con las de dos plásticos sintéticos comúnmente utilizados. Modificado (Zinn and Hany, 2005).

Propiedades	**PHA**			**PP**	**PS**
	scl-PHA	**mcl-PHA**	**lcl-PHA**		
Cristalinidad %	40-80	20-40	?	70	-
Punto de fusión (°C)	80-180	30-80	-	176	240
Densidad ($g \cdot cm^{-3}$)	1,25	1,05	?	0,91	1,06
Alargamiento de rotura (%)	6-10	300-450	-	400	1-60
Resistencia a la luz UV	buena	buena	buena	baja	baja
Resistencia a los disolventes	baja	baja	baja	buena	baja
Biodegradabilidad	buena	buena	buena	ninguna	baja

La variedad de composiciones y combinaciones de homopolímeros para formar copolímeros permite diseñar materiales con propiedades diferentes, permitiendo un abanico de posibles aplicaciones. Todas estas características dotan a estos materiales de un gran potencial para sustituir en varios casos a los plásticos convencionales (Visakh, 2015). En cuanto a su síntesis, es realizada forma natural por numerosas bacterias, como por ejemplo *Ralstonia eutropha* (recientemente llamada *Cupriavidus necator), Alcaligenes latus*, *Burkholderia sacchari*, y ciertas microalgas como *Botryococcus braunii* y *Spirulina* sp (Costa et al., 2019; Khosravi-Darani and Bucci, 2015).

Por otro lado, los mcL-PHA tienen una cristalinidad menor y los monómeros pueden incorporar grupos funcionales que permiten, tras su síntesis, modificar químicamente la estructura del polímero, confiriéndole nuevas propiedades. Presentan un punto de fusión y una temperatura de transición vítrea menores que los scl-PHAs o el polipropileno, y sus propiedades se acercan a las de los elastómeros termoplásticos. Los mcL-PHAs son sintetizados por algunos tipos de *Pseudomonas* fluorescentes como *Pseudomonas putida*; también determinadas bacterias como *Aeromonas hydrophila* y *Thiococcus pfennigii* sintetizan copolímeros en forma de scl

y mcl-PHA, como el poli(3-hidroxibutirato-co-3-hidroxivalerato-co-3-hidroxihexanoato) (P(3HB-co4HB-co-3HHx), poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate) (Khosravi-Darani and Bucci, 2015; Xie and Chen, 2008).

Respecto a los lcl-PHAs, son poco comunes y están menos estudiados que los otros tipos; aún no se ha detectado libremente en la naturaleza (Kunasundari and Sudesh, 2011). Sin embargo, hay trabajos donde se describe la producción de lcl-PHA, utilizando ácidos grasos de cadena larga y aceites como sustratos (ácido pentadecanoico, ácido heptadecanoico, ácido nonadecanoico, ácido heneicosanoico y aceite de palma) (Koller, 2018). También se han logrado sintetizar copolímeros scl-lcl-PHA bajo ciertas condiciones de cultivo (Singh and Mallick, 2008). Tanto los homopolímeros como los copolímeros presentan menores temperaturas de fusión y de transición vítrea que los otros dos tipos de polímero y así como una menor cristalinidad.

Aplicaciones de los PHAs

A pesar de que la comercialización y demanda mundial de los PHAs aún es pequeña, en la industria del embalaje y el envasado, el PHA se utiliza en la fabricación de películas de recubrimiento y recipientes para conservar de alimentos. En agricultura se emplea para la obtención de macetas, tubos de irrigación, plásticos de acolchado y matrices para el suministro controlado de pesticidas y nutrientes. También en la industria de los tintes se emplea como agente ligante en la formulación de pinturas a base de agua y en industrias que generan un gran impacto ambiental por el uso de materiales convencionales como redes de pesca, bolas de golf, etc. Por otro lado, debido a su biocompatibilidad, biodegradabilidad y baja citotoxicidad para las células también se utiliza en medicina y farmacia (suturas, soporte para ingeniería de tejidos, implantes temporales, material encapsulante, etc.) (Bugnicourt et al., 2014; Jindal and Tiwari, 2013; Zhang et al., 2018).

El diseño del material es algo importante a considerar porque características como la masa molecular la composición o el tipo de monómero afectan a

determinadas propiedades mecánicas del polímero. Estas características se pueden controlar mediante la manipulación de las condiciones de cultivo o químicamente, lo que expande los posibles usos de estos polímeros (Peña et al., 2014). Por ejemplo, el P(3HB) es muy frágil, por lo tanto, no se puede utilizar en muchos ámbitos, pero si se crean copolímeros de P(3HB) con otros PHAs sus propiedades cambian y el abanico de aplicaciones aumenta (Jindal and Tiwari, 2013).

2.3. Estado actual y desafíos para la comercialización del PLA y PHA.

En 2019, las capacidades de producción de PLA y PHA fueron de $294 \cdot 10^3$ y $25 \cdot 10^3$ toneladas respectivamente ("European Bioplastics," 2019). Para la próxima década, se prevé una tasa anual compuesta de crecimiento (CAGR, compound annual growth rate) de un 20,1 % para el PLA y un 6,3 % para el PHA, debido en buena parte a la creciente demanda de la industria del embalaje y el mercado de la salud (Al-Battashi et al., 2019). Además, esta tendencia se ve potenciada por las estrictas regulaciones contra los plásticos de un solo uso, el desarrollo sostenible y la economía circular. En este sentido, debido en buena parte a las acciones mencionadas anteriormente, Europa se convertirá junto a Asia-Pacífico (APAC, Asia Pacific) en el mercado clave a nivel mundial de polímeros de base biológica (Chinthapalli et al., 2019).

Como se ha visto anteriormente, tanto el ácido láctico (precursor del PLA) como el PHA y otros polímeros de base biológica se obtienen por fermentación, es decir a partir de microorganismos. El cultivo de microorganismos requiere de grandes cantidades de sustrato, especialmente de carbono y nitrógeno, cuyo precio es muy elevado. De hecho, su precio es el factor que tiene un mayor impacto en el coste de producción, llegando a representar un 40 % para el ácido láctico y hasta un 50 % en el caso del PHA (Plácido et al., 2017; Rodriguez-Perez et al., 2018). Otro factor que también afecta al coste de producción del PHA es su bajo rendimiento. Este hecho se debe a que el PHA (en comparación con el ácido láctico para producir PLA) se sintetiza en condiciones aeróbicas durante las cuales parte del carbono se pierde como CO_2 (Chen, 2009). Además, los procesos de extracción y purificación también

son muchas veces largos y laboriosos, lo que contribuye aún más a encarecer el producto (Wang et al., 2019). En el caso del PLA, la modificación química de las cadenas o la introducción de otros compuestos para mejorar sus propiedades puede afectar a su biodegradabilidad. Este factor hay que tenerlo en cuenta a la hora de su comercialización, ya que la gran ventaja de los plásticos biodegradables es que se degradan en el medio natural. De lo contrario estaríamos ante un material con las mismas características que uno convencional, pero más costoso (Sidek et al., 2019).

En el futuro, se prevé que los problemas asociados al alto coste de la mayoría de los bioplásticos sean menos importantes, porque se espera un aumento de los precios del petróleo. Sin embargo, las influencias económicas por sí solas no impulsarán el auge de esta tecnología. Será necesario mejorar y desarrollar los procesos actuales, además de seguir buscando nuevas materias primas renovables que posibiliten una producción sostenible. También será necesario por parte de gobiernos y empresas contabilizar el ahorro que supone, en aspectos medioambientales y de salud, la utilización de bioplásticos, así como el uso de desechos como materias primas para su síntesis.

2.4. Producción de ácido láctico y PHA utilizando fuentes de carbono alternativas.

La búsqueda de sustratos alternativos y con menor coste se ha convertido en algo esencial para el desarrollo sostenible de estas tecnologías. Desde la perspectiva de la bioeconomía circular, hace tiempo que se comenzó a explorar la posibilidad de utilizar desechos orgánicos como fuentes de carbono y nitrógeno sostenibles, pero es hoy en día, con el auge de los plásticos biodegradables, cuando realmente se ha se ha empezado a estudiar su potencial. Estos residuos incluyen los desechos agrícolas, de bosques y jardines, así como los desechos de alimentos y papel.

Los desechos comúnmente utilizados como fuente de carbono a gran escala (bagazo de caña de azúcar, jarabe de maíz, y paja de trigo, etc.) no siempre están disponibles cerca de las plantas de producción de PLA y PHA, y el transporte de estos residuos a los puntos de fabricación contribuye a la contaminación global (Shahzad et al., 2013). Por el contrario, los residuos sólidos urbanos (MSW, municipal solid

waste) se generan en grandes cantidades en todas las ciudades y su fracción orgánica (OF, organic fraction) está formada mayoritariamente por materiales biodegradables (azúcares, celulosa, hemicelulosa, almidón, proteínas) y minerales (Campuzano and González-Martínez, 2016). En España anualmente se producen 877.000 toneladas de fracción orgánica de residuo sólido urbano (residuos domésticos y de jardín), de las cuales el 2 % se generan en el País Vasco. Este tipo de residuos se recogen de manera selectiva y el 70 % es procesado para la producción de compost o biogás, mientras que el resto (30 %) es eliminado en vertederos o incinerado (MITECO, 2017). A pesar de que la mayor parte de la fracción orgánica se deriva la generación de compost, a veces su baja calidad puede dificultar su comercialización, dando lugar a una sobreproducción (Cesaro et al., 2015; Doña-Grimaldi et al., 2019).

La alta y continua disponibilidad tanto de la fracción orgánica de residuo sólido urbano y de su compost, así como su gran contenido en nutrientes (especialmente carbono y nitrógeno), hacen que ambos sustratos sean excelentes para la fabricación de productos con un alto valor añadido. De hecho, hay varias investigaciones en las cuales a partir de la fracción orgánica de residuo sólido urbano se obtuvieron hidrolizados ricos en carbono, que más tarde se utilizaron como sustrato en la producción fermentativa de lípidos, ácido láctico, butanol, etanol, ácido succínico y biogás, etc. (Ghanavati et al., 2015; López-Gómez et al., 2019; Matsakas et al., 2017). Usar de este tipo de sustratos, en la fabricación de estos productos no solo es una forma de disminuir la dependencia de recursos no renovables, sino que también es una manera de reducir la cantidad de desechos orgánicos y de plásticos que de otra manera acabarían agravando los problemas de contaminación ya existentes.

HYPOTHESIS AND OBJETIVES

HIPÓTESIS Y OBJETIVOS

HYPOTHESIS AND OBJETIVES

Biodegradable bioplastics are emerging onto the market as sustainable and environmentally friendly materials. Nevertheless, their high production cost is one of the limitations for their use as substitutes for conventional plastics. Reducing that cost and increasing the yield is a great challenge, since these would improve the market and applications of these polymers. In this sense, within a circular bioeconomy strategy, the use of the organic fraction of urban solid waste (OFMSW) as raw material is presented as an opportunity that could contribute to increasing environmental, economic and social sustainability. Taking these aspects into account, the present work is based on the following hypothesis:

The use of organic waste, such as OFMSW or its derived compost, in the production of high-value compounds can be a good strategy to i) reduce the amount of municipal waste that ends up in landfills and ii) reduce production costs and iii) encourage the development of a circular economy.

In this context, the main objective of this doctoral thesis is to explore the use of the organic fraction of urban solid waste, and the compost derived from it, as possible sources of carbon and nitrogen for the production of compounds with high added value. Based on the growing interest in and demand for biodegradable plastics, the compounds selected were P(3HB) and lactic acid (precursor of PLA).

For this purpose, three studies were proposed. Two of them focused on the use of the sugars present in municipal solid waste to produce P(3HB). The third focused on the use of the protein-like compounds, present in compost obtained from the organic fraction of urban solid waste, to produce lactic acid. Experiments took place mainly in Neiker, however those corresponding to the growth and production tests with *Burkholderia sacchari* DSM 17165 were carried out at the Instituto Superior Técnico of Lisbon, and correspond to the international predoctoral stay.

To achieve the central objective of this doctoral thesis, the following specific objectives were proposed.

Study 1. Assessing the organic fraction of municipal solid waste as carbon source for P(3HB) production.

1. To design and optimize a strategy to obtain sugar-rich hydrolysates using the organic fraction of urban solid waste as feedstock (**Manuscript 1**).

2. To evaluate the hydrolysate obtained as substrate for P(3HB) production by *Burkholderia sacchari* DSM 17165 (**Manuscript 1**).

Study 2. Scaling-up of poly (3-hydroxybutyrate) production by fed-batch culture of *Burkholderia sacchari* from municipal solid waste hydrolysates and plum waste juices.

1. To scale the process on a laboratory scale (5 L bioreactor) using a plum concentrate as feed during fermentation (**Manuscript 2**).

2. To evaluate the economic and environmental feasibility of the process, including polymer extraction (**Manuscript 3**).

Study 3. Assessing the organic fraction of municipal solid waste compost as nitrogen source for lactic acid production.

1. To develop a process to produce a nitrogen-rich (amino-acids) concentrate from OFMSW compost (**Manuscript 4**).

2. To evaluate the possibility of using the concentrate as a culture media to cultivate lactic acid bacteria (*L. Fermentum* ATCC 9338 and *L. Plantarum* NCIMB 8826) in order to produce lactic acid (**Manuscript 4**).

EXPERIMENTAL DESIGN

DISEÑO EXPERIMENTAL

EXPERIMENTAL DESIGN

In order to achieve the proposed objectives of this PhD Thesis, the experimental design, more widely explained in the material and methods section of each manuscript, was as follows.

The organic fraction of municipal solid waste was selectively collected from the municipalities of The Basque Country. Then it is transported to the EPELE composting plant (Gipuzkoa, Spain). This fraction is made up of domestic waste mixed with garden waste as structuring material. After removing the residual impurities (e.g., small stones, plastics, glass and metals), the mixture is homogenized and placed in composting tunnels. During the composting process its composition in macronutrients varies depending on the maturation stage of the compost. Thus, in the initial phase, the residue is richer in polysaccharides, while in the final phase it is richer in protein-like compounds. The composition of each phase can also vary according to the time of year or the geographical area. Therefore, to make the results of all studies comparable, the samples were taken on the same date (November 2017).

For each study, different phases of the composting process were used, depending on the substrate to be obtained. The initial phase was used to obtain sugar-rich substrates, while the final phase was employed for amino-acid rich substrates.

Study 1. Assessing the organic fraction of municipal solid waste as carbon source for P(3HB) production.

For the first study, the initial phase of the composting process was used. Waste samples were collected in November 2017 from the local composting plant EPELE. The biomass was screened, characterized and finally dried to preserve it better. To release the sugars, a two-step process was developed. The first step consisted in a diluted acid pre-treatment, in which 13.5 %; w/v of biomass was mixed with 0.18 $mol \cdot L^{-1}$ H_2SO_4 solution and heated at 121 °C for 60 min. In the next step, the pre-

treated biomass was enzymatically hydrolysed using an enzyme cocktail of Pentopan 500 BG, Celluclast BG and Glucoamylase NS 22035. To maximize the release of sugars, the influence of some enzymatic hydrolysis variables was studied. The hydrolysate obtained was supplemented with salts and extra glucose, and tested in shake flask assays to produce P(3HB) by *Burkholderia sacchari* DSM 17165. (figure 11).

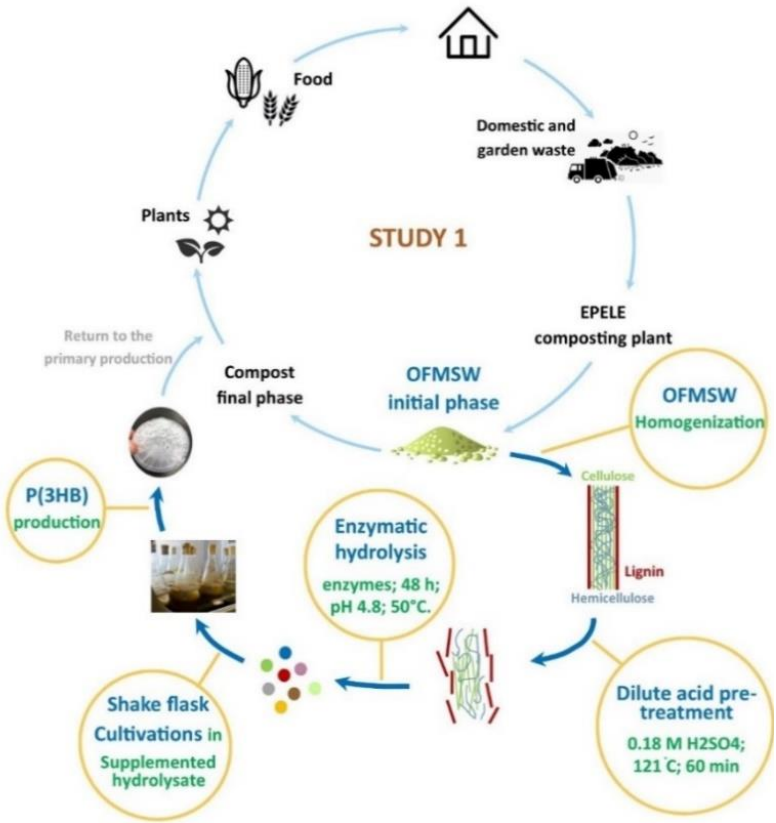

Figure 11: Schematic diagram of the first study. Part highlighted in dark blue represents the steps carried out in this study.

Study 2. Scaling-up of poly(3-hydroxybutyrate) production by fed-batch culture of *Burkholderia sacchari* from municipal solid waste hydrolysates and waste plum juices.

In the second study, the process was scaled-up using, as a starting point, the data obtained in the first. To release the sugars, the same method was used, which consisted of pre-treatment with diluted acid followed by enzymatic hydrolysis. P(3HB) production was carried out in 5 L bioreactors operated in fed-batch mode, using the hydrolysate as growth medium and a plum concentrate as feed. The information collected from the hydrolysis and fermentation steps served to perform a techno-economic evaluation of P(3HB) production process from OFMSW (figure 12).

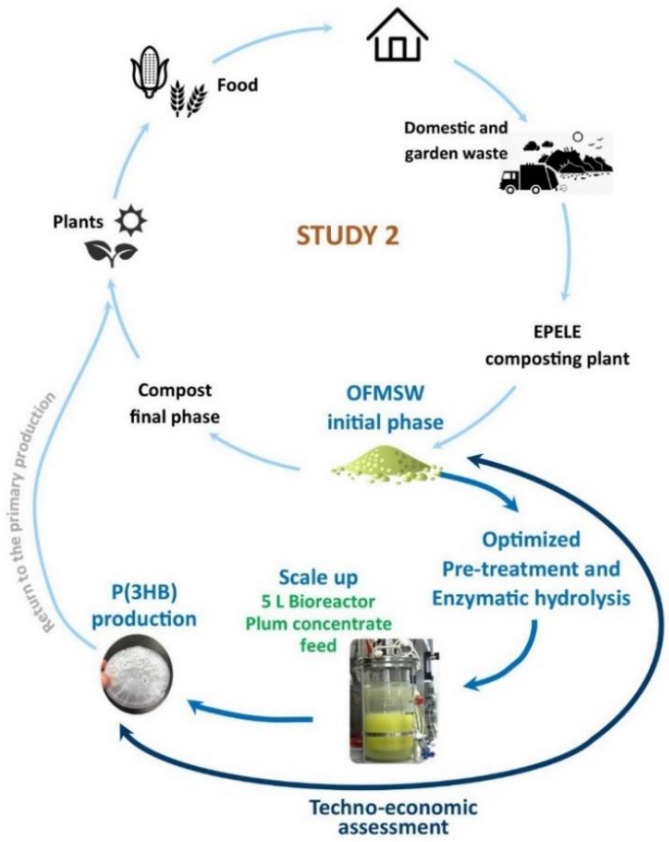

Figure 12: Schematic diagram of the second study. Part of the figure highlighted in dark blue corresponds to the work carried out in this study.

Study 3. Assessing the organic fraction of municipal solid waste compost as nitrogen source for lactic acid production.

A third study was conducted using the final phase of the composting process. Compost samples were collected in November 2017 from EPELE composting plant. The biomass was screened, dried and chemically characterized. To release the amino-acids from the compost, enzymatic digestion was carried out. The hydrolysis was based on the combined action of glucanase Viscozyme L endoprotease Alcalase 1.5 MG and exoprotease Flavourzyme 500 MG. The amino-acid rich hydrolysate was supplemented with glucose and salts, and turned out to be quite suitable for the production of lactic acid by *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB 8826 (figure 13).

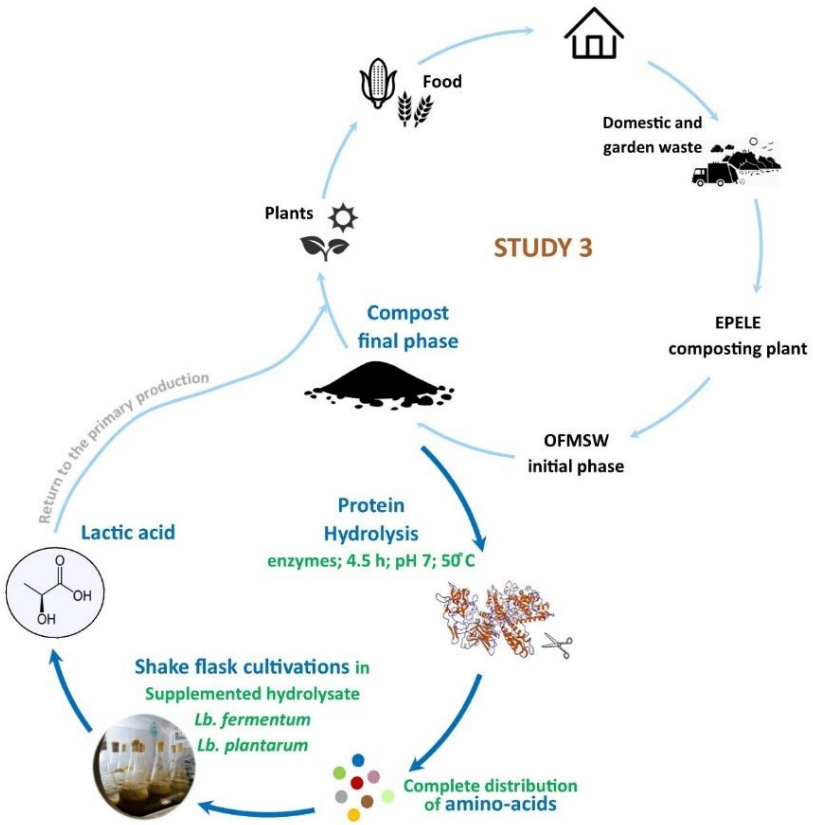

Figure 13: Schematic diagram of the third study. Part of the figure highlighted in dark blue corresponds to the work carried out in this study.

SUMMARY

RECAPITULACIÓN

SUMMARY

As was previously mentioned in the introduction to this Doctoral Thesis, given the growing environmental and health problems caused by increased municipal waste production, especially organic fraction and plastics, scientific researchers are focusing on the search for new solutions compatible with existing ones. These solutions involve the adoption of circular economy models which prioritise reuse, reduction and use of biodegradable materials.

Within a circular economy model, this research focused on the use of waste for the production of two products with high added value. On one hand, the organic fraction of municipal solid waste (OFMSW), rich in polysaccharides, was used as carbon source for the production of a biodegradable bio-plastic (poly(3-hydroxybutyrate)); while on the other, the compost derived from the OFMSW, rich in protein like compounds, was used as nitrogen source for the production of lactic acid.

This work can be divided into three main parts. The first part aimed to assess the possibility of using the OFMSW for the production of poly(3-hydroxybutyrate) (P(3HB)). In the second part, the process already validated in the first part, was scaled-up to 5 L bioreactors, in addition the process was economically and environmentally evaluated. Finally, the third part focused on solving the problem generated by the accumulation of OFMSW compost through its use as a nitrogen source in the production of lactic acid (LA), precursor of polylactic acid (PLA).

A) Assessing the organic fraction of municipal solid waste as carbon source for P(3HB) production.

In this section, we investigated the possibility of using OFMSW as sugar platform for the production of a bio-polymer (P(3HB)). The results of this study were collected and discussed in **manuscript 1**.

Municipal solid waste (MSW) is generated massively in all cities around the world. Plastic materials, paper, glass and the organic fraction stand out in its composition. The latter represents the highest portion of municipal solid waste. How to manage the residues is crucial to prevent risks of both pollution and to health. However, current management systems are not efficient enough to prevent some of these polluting materials from reaching ecosystems. As a result, the environmental impact of this waste, particularly of the most persistent, i.e. plastics, is overly high and has serious consequences.

As an alternative to existing management systems, strategies based on reuse and recycling have emerged, which are framed within a circular bioeconomy model. Accordingly, following a circular economy strategy would allow that these materials, considered waste, to have a second life as feedstock in obtaining new high-value products. This approach would not only reduce the cost of raw materials, but also avoid the problems caused by the accumulation of waste. In this line, several studies have investigated the use of the organic fraction of municipal solid waste to obtain high-value compounds such as lipids, lactic acid, butanol, ethanol, succinic acid and biogas among others (Farmanbordar et al., 2018; Ghanavati et al., 2015; López-Gómez et al., 2019; Matsakas et al., 2017).

In the present study, the organic fraction of municipal solid waste, which was mainly composed of domestic and garden residues, was evaluated as sugar platform for the biological production of biodegradable bioplastic poly(3ydroxybutyrate) (P(3HB)). At the molecular level, the three major components of OFMSW are cellulose, hemicellulose and lignin (47 %). Cellulose is the most abundant of the three macromolecules and is only formed by D-glucose units. Hemicellulose, by contrast, contains several sugar monomers, of which xylose stands out. Lignin consists in cross-linked phenolic polymers. Of these, only cellulose and hemicellulose contain fermentable sugars. However, their complex structure is a great barrier for access to sugars by microorganisms. To transform the biomass into fermentable sugars it is necessary for it to be treated. Of these treatments, acid hydrolysis is the main technology to recover monosaccharides from lignocellulosic

biomass, since it is cheap, effective and does not require complicated processes (Lenihan et al., 2010; Loow et al., 2016). Nevertheless, the use of concentrated acid solutions may generate inhibitory compounds, which could affect fermentation (Behera et al., 2014). In the current study, to avoid the formation of unwanted compounds a combined process of thermo-chemical pre-treatment with dilute acid and an enzymatic hydrolysis was performed. The dilute acid pre-treatment helped to break down the structure of cellulose, hemicellulose and lignin, while minimizing the generation of toxic products, and facilitated access to the enzymes in the enzymatic hydrolysis step. The combination of both thermochemical pre-treatment and enzymatic hydrolysis minimized the generation of toxic compounds but the high cost of the enzymes, compared with the sole use of sulphuric acid, would affect to the process viability. A possible alternative could be the use of bases instead of dilute acid. Alkaline treatments are usually less effective in extracting sugars, but they generate a lower load of toxic compounds for the cells. Among the most widely used bases include sodium and potassium hydroxide, calcium hydroxide and ammonia (Jönsson and Martín, 2016; McIntosh and Vancov, 2010).

In the combination of thermochemical pretreatment and enzymatic hydrolysis, the latter plays an important role, since enzymes are responsible for breaking the bonds that hold monosaccharides together, releasing them into the broth. Therefore, the selection of a suitable enzymatic cocktail and the optimization of the parameters that affect hydrolysis are necessary to obtain good hydrolysis yield. In our study, the enzyme cocktail was selected based on the polysaccharide composition of the waste. Thus, since the residue was composed of cellulose and hemicellulose, the enzymatic preparations Pentopan 500 BG and Celluclast BG were used. Pentopan is composed of a xylanase which acts on hemicellulose xylans, while Celluclast is a mix of three enzymes that act on cellulose. By pretreating with dilute acid and under optimal enzymatic hydrolysis conditions, 49 % of the sugars contained in the biomass were released. If this result was compared with those obtained by other authors, it can be observed that the hydrolysis yield is not very high (Li et al., 2012; López-Gómez et al., 2019). However, it was possible to increase the yield up to 56 %, by adding

another enzyme, which acted on the small portion of starch contained in the waste. Finally, the principal monosaccharide in the hydrolysate was glucose (21 $g \cdot L^{-1}$), but xylose (4 $g \cdot L^{-1}$) and arabinose (0.6 $g \cdot L^{-1}$) were also obtained. This sugar concentrations were in broad agreement with those observed by other researchers when they hydrolysed the OFMSW (Ghanavati et al., 2015; Li et al., 2012; Mahmoodi et al., 2018). The amount of sugars obtained was sufficient to create a culture medium, taking into account that the initial concentration of the medium in fermentation processes to produce P(3HB) is between 10 $g \cdot L^{-1}$ and 40 $g \cdot L^{-1}$ (Al-Battashi et al., 2019; Colombo et al., 2017; González-García et al., 2019).

In a fermentative process, the selection of the type of microorganism is very important because fermentation viability will largely depend on it. There are several factors that can influence the selection of the strain. These factors are productivity, growth time, pathogenicity, and tolerance to toxic compounds, among others. In this case, in order to use the hydrolysate as a carbon source, a microorganism with the ability to consume several types of monosaccharides simultaneously is necessary. Usually, microorganisms that live in sugar-rich environments assimilate for preference the one that provides the most efficient growth. This preference for one sugar is also called carbon catabolite repression (CCR) (Al-Battashi et al., 2019; Vinuselvi et al., 2012). CCR can represent a problem when the fermentation substrate is composed of a sugars mixture, such as in the OFMSW hydrolysate, because the preferential consumption of one sugar can result in the accumulation of the others. If one sugar is consumed over the others these accumulate in the medium and may inhibit the growth of the microorganism. This effect is significant when the fed-batch cultivation strategy is applied.

Taking this information into consideration, our study selected a microorganism able to accumulate P(3HB) upon consumption of glucose, xylose and arabinose. This was *Burkholderia sacchari* DSM 17165, a strain isolated from the soil of sugar cane plantations in Brazil. *B. sacchari* is a gram-negative bacterium with enormous potential to produce large amounts of P(3HB) from lignocellulosic feedstocks, as was

demonstrated Cesário et al. (2014), Lopes et al. (2014), (Obruca, 2015) and (González-García et al., 2019).

OFMSW hydrolysate has a complete nutrient composition, where in addition to previously mentioned sugars, other minerals such as nitrogen, calcium and magnesium stand out. This was a positive factor, since its high nutritional value facilitated its use as a culture media. When the OFMSW hydrolysate was used as culture media with *B. sacchari*, the cell growth was similar to that of the control, but the accumulation of P(3HB) did not occur or was very low. This effect was due to the fact that *B. sacchari* and other strains only produce P(3HB) under unbalanced growth conditions, that is to say in an excess of carbon and a simultaneous limitation of nutrients such as nitrogen, phosphorus, sulfur, magnesium or oxygen (high carbon/limiting nutrient ratio) (Alves et al., 2017). The results obtained in this study indicated that the culture media used was not limited. In fact, it was noted that at the start of the cultivation, the nitrogen concentration was very high and, therefore the C/N ratio was very low (7.4). According to Lopes et al. (2014) and Al-Battashi et al. (2019), C/N ratios of 20 or higher are necessary for polymer accumulation. To solve this problem, the hydrolysate was supplemented with extra glucose after exhaustion of the initial carbon, so, as a part of the nitrogen had also been consumed, the C/N ratio increased. The strategy adopted turned out to be suitable since there was production of P(3HB). However, it did not begin until the C/N ratio was high enough and, the yield of polymer on sugar consumed (0.13) was not as high as that obtained with the control media (0.20). The cause of this effect was probably due to the delay in P(3HB) production and also to the hydrolysate favoring biomass over polymer production. This was in agreement with the findings of Al-Battashi et al. (2019), who also obtained higher cell biomass when they replaced simple sugars by a hydrolysate. Finally, the production of P(3HB) (4.92 $g \cdot L^{-1}$) after supplementing the hydrolysate with glucose and salts was comparable to that obtained by other authors under similar conditions (Al-Battashi et al., 2019; Cesário et al., 2014; Silva et al., 2004).

In this case although the excess of nitrogen can be considered an inconvenience when working at larger scales, having an initial medium with a high nitrogen content can be an advantage. This is because in order to obtain high productions of P(3HB), high concentrations of cells should be reached during the initial phase of the culture, and nitrogen is essential for cell growth.

B) Scaling-up of poly (3-hydroxybutyrate) production by fed-batch culture of *Burkholderia sacchari* from municipal solid waste hydrolysates and waste plum juices.

The second part of my Doctoral Thesis was based on the data obtained in the previous study (Assessing the organic fraction of municipal solid waste as carbon source for P(3HB) production), to carry out the scaling-up of the process (**manuscript 2**). The techno-economic and environmental evaluation of P(3HB) production from OFMSW was also performed in **manuscript 3**.

Evaluation of the industrial production of a process requires the study of its scale-up. This involves going through it step by step and optimizing the process, first in shake flasks, then on a laboratory and pilot scale and finally on an industrial scale (Cuello et al., 2016). Shake flask assays, like those carried out in the first study, are essentials in the design of a fermentation process. The simple design of the shaking flask allows for many tests, even simultaneously, without significant resource expenditure. This helps to make a preliminary assessment of whether the process is feasible.

The present study focuses on the scaling-up of the P(3HB) production from shake flasks to a 5 L stirred tank fermentor. However, moving from shake flasks to laboratory scale fermentor is a significant challenge, since both systems are quite different in geometry, mass transfer, mixing, heat dissipation, among others. All these differences can influence the behavior of the culture. Therefore, although the process may be feasible at shake flask level, it may not be at other levels (Berenjian, 2019). The bioreactors or fermentors, such as those used in this study, are systems that are equipped with sensors, mechanical agitation and ports for the supply of

gases and liquids. In addition, they are associated to a software that allows real-time monitoring of fermentation. This precise control provides a lot of information, which is very useful when it comes to improving the process.

Of all the modes of carrying out a fermentation, the batch mode is the simplest. Indeed, it has been widely used in the biotech industry (Xin et al., 2018). During the batch process, the culture media and the producing microorganism are added to the system at the same time and the fermentation continues until the nutrients in the medium are exhausted. The biggest disadvantage of working in this mode is that the achievable cell densities are not overly high (Xin et al., 2018). Thus, this mode is generally used as a starting point to optimize conditions in the early stages of experimental design. If higher productivity is required, or high substrate concentrations are toxic to the microbial culture (catabolism repression), working in fed-batch mode can be a good alternative. In fed-batch mode microorganisms grow in batch mode until the substrate is depleted, then nutrients are added to the fermentor in increments until the end of fermentation (EL Moslamy, 2019). This slow and gradual supply of nutrients prevents their accumulation in the medium, from reaching levels that could be toxic to cells.

The strain used in this study (*Burkholderia sacchari* DSM 17165), as well as most P(3HB) producing strains, is sensitive to high concentrations of the substrate (Raposo et al., 2017). Therefore, it is preferable that the culture medium is not highly concentrated. Moreover *B. sacchari* shows a differentiated growth and production profile. This is to say that when there is an abundance of nutrients, cell density increases rapidly (growth phase), but when there is a limitation in some of its essential nutrients and at the same time an excess of carbon, the cells begin to accumulate P(3HB) (accumulation phase) (Anjum et al., 2016; Schwarz et al., 2018). This characteristic profile and the difficulty of using concentrated mediums makes operating in fed-batch mode the most suitable, which favours increased cell density, while in the accumulation phase carbon-rich feeding is required. In this regard, the OFMSW hydrolysate studied in the first part of this Doctoral Thesis, showed a comprehensive nutrient profile, which made it suitable for its use as initial medium.

Nevertheless, its sugar concentration (glucose, 21 $g \cdot L^{-1}$; xylose, 4 $g \cdot L^{-1}$ and arabinose, 0.6 $g \cdot L^{-1}$) was not high enough to be used as a feed solution. This is because a feed solution must be highly concentrated to avoid dilution of the culture. Thus, using OFMSW hydrolysate as a feed solution would imply increasing its concentration of sugars, and for that it would be necessary to remove a portion of its water content. But this additional step would involve high economic and energetic costs. As an alternative, some authors have had the idea of using fruit waste juices as carbon source in the production of different products (Diboune et al., 2019; Ravindran and Jaiswal, 2016; Zabed et al., 2014). These types of substrates tend to have a high sugar concentration and do not need to be concentrates. In our study, following a similar strategy, we used the OFMSW hydrolysate, rich in nutrients, as initial medium and a plum concentrate, rich in carbon (120 $g \cdot L^{-1}$ glucose and 60 $g \cdot L_{-1}$ fructose), as feed solution.

Operating in fed-batch mode requires continuous supply of carbon. The strategy used for the addition of carbon largely depends on the type of process, the microorganism or on the product to be obtained. In the production of P(3HB) by *B. sacchari*, the addition of feed is typically carried out on the basis of DO-stat, that is, based on the dissolved oxygen in the culture medium (Cerrone et al., 2015; Raposo et al., 2017). For this purpose, the feed solution is added to the culture media when a constant increase in the value of dissolved oxygen (DO) is detected, which indicates the exhaustion of the carbon source. The long duration of fermentation requires that the addition of substrate be automatic, which necessitates automating the process. Automated feeding is normally carried out by programming the fermenter to add the feed based on a parameter related to fermentation, such as dissolved oxygen. For example, Cesário et al. (2014) associated the addition of substrate to the stirring speed, which in turn depended on dissolved oxygen. In our study, the limitation to programming the equipment made it impossible to automate feeding based on agitation. As an alternative, we programmed the substrate additions using a plug-in timer. To determine the rate of carbon consumption and therefore program the feed

addition, preliminary fermentations were conducted in which the rate of carbon consumption were studied.

The selection of this feeding mode together with the use of OFMSW hydrolysate as initial culture medium and plum concentrate as feed solution, turned out to be a good strategy for the production of P(3HB). However, the values of productivity (0.59 $g \cdot (L \cdot h)^{-1}$) and accumulation (43 %) obtained were generally lower than those reported by other authors (Cesário et al., 2014; Pradella et al., 2010; Silva et al., 2004), who also used hydrolysates as carbon source. This difference may be due to the fact that OFMSW is composed of residues of different nature, which make it very heterogeneous, while they used simple lignocellulosic residues of a single type. In fact, the values of cell growth (71 $g \cdot L^{-1}$) and accumulation of P(3HB) (43 %) obtained were very similar to those obtained by other authors with complex biomasses (Al-Battashi et al., 2019; Cerrone et al., 2015). Another factor that may also have influenced productivity is the use of the plug-in timer, since programming the addition of feed based on the data obtained in previous fermentations may not be as effective as doing it based on oxygen demand. This is because the fermentation process does not always advance in the same way and it is possible that the feed demand of one culture may not overlap, in time, with that of another culture, generating a gap that can accumulate in successive additions. It would be necessary to study this effect and find out if it negatively influences the development of fermentation.

Looking ahead, it would be appropriate to investigate the possibility of detoxifying the OFMSW hydrolysate to attempt to increase the yield of the fermentation. Another option could be a local area search for other types of waste which are rich in sugars but less complex, and combine them with OFMSW.

Regarding the economic aspect, the production cost of biodegradable bioplastics and specifically of P(3HB) is significantly higher than that of petroleum-based plastics. This is largely due to the fact that the industry responsible for manufacturing conventional plastics has had a longer journey and, in addition, its

production capacity is broader. Since the production of P(3HB) is carried out through fermentation processes, the carbon source represents one of the highest production costs. In addition to the carbon source, there are other factors such as fermentation performance, equipment sterilization, discontinuity of processes and product extraction that also influence the increase in production costs (Wang et al., 2014).

Making an estimate of the cost of producing the target product is really important for the development of the process on an industrial scale. In this regard, the easiest way to estimate the viability of a process is through its techno-economic evaluation. By a techno-economic analysis it is possible to determine the consumption of materials and energy (material and energy balances) throughout the process, the cost of production of the product, the total capital cost, the annual production cost, as well as calculate its profitability. The techno-economic evaluations are based, on one hand, on process data obtained in laboratory-scale steps, and on the other hand, on equipment and consumable price databases, among others. Costs associated with labor, waste generation rates and gas emissions are also taken into account. The profitability of the process is evaluated with a number of indicators, namely gross and net profit, gross margin, return on investment (ROI), net present value (NPV) and payback time.

In this study, the viability assessment of the P(3HB) production process from OFMSW was carried out, with the aid of the SuperPro Designer® software. To carry out this evaluation, an industrial plant was simulated, whose construction would take place in The Basque Country. The plant would have a processing capacity of 1 tonne·day^{-1} of OFMSW and would operate for 330 days·year^{-1}. The simulation considered two scenarios. The first scenario was based on the laboratory-scale process outlined in manuscript 2, and consisted of three steps. The first step included the part of the process focused on releasing sugars from OFMSW. The second step focused on fermentation, where the sugar-rich hydrolysate, obtained in step 1, was used as the initial culture medium. The nutritional requirements of the culture during fermentation (fed-batch phase) were covered by adding a carbon-rich

plum concentrate. The third step consisted in the extraction of P(3HB) from inside the cells. In the second scenario, instead of using the OFMSW hydrolysate as initial medium, a basal medium supplemented with glucose was used. Carbon demand during fermentation, as in the first scenario, was also covered by the plum concentrate. Therefore, this scenario only considered steps 2 and 3, both being the same as in scenario 1. The approach of these two scenarios served to determine if the use of OFMSW, as initial medium, would be a good alternative to decrease the cost of production of P(3HB), compared to the use of basal media supplemented with glucose.

The evaluation of both scenarios revealed that the production cost of P(3HB) in the first was approximately 28 % higher than in the second. This difference was mainly due to the number of steps of the process, since scenario 1 had an additional step (step 1). This step was intended to extract the sugars from the OFMSW and for this purpose it was necessary to purchase a larger amount of equipment (sterilizer, mixing tank, reactor, etc.). The energy consumption of this equipment also contributed to the increase in the cost of scenario 1. Other causes that influenced the extra cost of the first scenario were the price of the enzymes needed to extract the sugars and labor, since in this scenario a greater number of workers was required. It is clear that the use of the hydrolysate alone as an initial medium did not compensate for the cost of its production, since the carbon required to start the fermentation (start of step 2) only represented a small percentage of all the carbon used in the process. In fact, the highest amount of carbon was provided to the culture during the fed-batch phase by means of the plum concentrate. It is possible that the use of OFMSW hydrolysate as the sole carbon source was cheaper than the use of glucose. However, this option was rejected in this study, because for the proper use of the hydrolysate as a feed-solution, it should have a much higher concentration of sugars. Increasing its concentration would imply the elimination of water and therefore an increase in energy consumption.

As regards the profitability of the process, the analysis of the indicators, mentioned above, showed that neither of the two scenarios was economically viable.

In fact, the estimated P(3HB) production costs turned out to be higher than those reported by other authors in similar processes (Lam et al., 2014; Nieder-heitmann et al., 2019; Quintero et al., 2013). Although the production of the hydrolysate supposed a high cost, there were more factors that influenced the infeasibility of both scenarios. The most important factor was the low fermentation yield, both in terms of biomass production (71 $g \cdot L^{-1}$) and P (3HB) accumulation (45 %). This fact has been previously discussed (manuscript 2) and is probably due to the complexity of the substrates (OFMSW hydrolysate and plum concentrate). Another factor could be the consumption of solvent in the extraction step. However, its impact was not significant, since its reuse from one cycle to another was considered.

In spite of the proposed process not being profitable, it would be possible to improve its economic viability by taking advantage of the waste generated during the process. In this respect, the remaining biomass after polymer extraction step could be reused for the production of other types of high-value products such as biogas, H_2 and proteins, electricity, among others, as other researchers have suggested (Dávila et al., 2016; Kwan et al., 2015; Nieder-heitmann et al., 2019). CO_2 generated during the fermentation could also collected for its sale (Sadhukhan et al., 2016). The solid phase of the OFMSW hydrolysate, which still contains cellulose and hemicellulose, could serve to produce hydroxymethylfurfural (HMF), luvulinic acid and other compounds that are appreciated by the industry for the synthesis of new materials (Hayes and Becer, 2020; Kohli et al., 2019). An increased scale could also contribute to improving profitability, as evidenced by Choi, (1999). Other aspects that could also improve the profitability of the process would be to increase the efficiency of the fermentation, to look for a microorganism that is better adapted to complex substrates such as OFMSW hydrolysate, or to investigate new feed strategies that generate greater amounts of biomass.

Techno-economic evaluations, such as the one carried out in this study, tend to only consider production costs at the plant while other costs generated during obtaining raw materials and the elimination of the residues, and that are related to the damage to the environment or the increase of diseases, are not taken into

account. Although these costs are not evaluated, they can represent a high percentage of the true cost of the product (Hanley et al., 2019). The simplest way to consider these costs, which are not direct but are related to the manufacture of the product, could be through taxes or fiscally favoring the low-impact products. These strategies are already applied for example with CO_2 emissions, where companies or countries pay a fee for the amount of gases they emit (Bachmann, 2020). In this case, using waste as raw material and producing a material that is completely biodegradable generates much less impact. Therefore, if costs related to damage to the environment or human health were taken into account, the production of bioplastics could become more profitable than the production of petroleum-derived plastics.

Environmental evaluation of the P(3HB) production process showed that the greatest environmental impacts generated by the plant were associated with the use of the extraction solvent (anisole), and the burning of fuels for power generation. The fact of not using toxic compounds helped that the potential environmental impact was, in general, very small. If both scenarios are compared, scenario 1 had a greater impact due to the OFMSW sugar release step (step 1). This step, only present in the first scenario, required large amounts of energy.

Moreover, this type of process, based on a circular bioeconomy, where waste is used as raw materials and sustainable materials are also produced, may generate lower impacts than does the production of materials of non-renewable origin. Therefore, it is logical to think that, as legislation gradually toughens, companies will choose to produce materials like those shown here.

C) Assessing the organic fraction of municipal solid waste compost as nitrogen source for lactic acid production (manuscript 4).

The third part of my Doctoral Thesis focusses on study the possibility of using OFMSW derived compost for the production of high-value lactic acid compound. The results obtained from this study are in **manuscript 4**.

As seen above, the organic fraction represents a high percentage of municipal solid waste. Among all the methods of recycling organic waste, composting is one of the most used. Some benefits of transforming a waste into a product such as compost are reducing greenhouse gas emissions, avoiding contamination of water sources, and reducing the spread of diseases among others (Sánchez et al., 2015). However, for safe production of compost, quality standards must be met, even more so when complex waste, such as the organic fraction of urban solid waste (OFMSW), is used. Current European frameworks that assess compost quality only take into account certain physical-chemical parameters, pathogen content assessment and seed germination. There is no thorough regulation. The lack of any rigorous European regulation forces, countries to somehow have their own regulations to assess the quality of compost. This implies that the levels of demand are not equal and therefore hinders its marketing (Cesaro et al., 2015).

High-quality compost is usually highly valued, while when it does not reach a minimum standard there are serious difficulties for its commercial use. This problem often causes excessive stocks in production plants or leads to the use of compost in applications where its quality is not important, such as landfill covering, urban gardening, landscaping, soil stabilization, energetic valorization, among others (Doña-Grimaldi et al., 2019; Ghosh, 2019; Torquebiau, 2016). In addition to the quality factor, in The Basque Country there are other factors that reduce its viability in the market, which include the scarcity of agricultural soils, the surplus of livestock residues that are most in demand and the difficulty of mechanically distributing it in the cultivation fields (Diputación foral de Gipuzkoa, 2016). It is clear that all these factors, which have been previously set out, have a negative influence on the demand for compost, therefore it is necessary to find an alternative solution to its over-accumulation.

Following a circular bioeconomy strategy, similar to that used with OFMSW for the production of P(3HB), the use of compost as feedstock in the production of high-value products can be assessed. OFMSW compost is characterized by having a high content of organic nitrogen where protein-like compounds stand out (16 %). These

compounds are generated during the maturation process of the compost by microbial activity (Matsunaga et al., 2013; Pichler et al., 2000). In the present study, we evaluated the possibility of releasing the amino acids that were part of the compost proteins to create a nitrogen-rich culture medium; and use it in the production of value-added products, through the cultivation of microorganisms.

Usually, the release of amino acids involves a hydrolysis process, which can be chemical or enzymatic. In this case, we chose to use enzymes, since the conditions were milder and thus the degradation of amino acids was avoided. The enzymatic process used to release the amino acids was based on the work of Romero García et al. (2012) and consisted in the use of three different enzymes, a glucanase (Viscozyme L), an endoprotease (Alcalase 1.5 MG) and an exoprotease (Flavourzyme 500 MG). Glucanase was added at the beginning of the process to decrease the viscosity of the medium and favor the access of the proteases to the proteins, followed by endoprotease and finally exoprotease was added. The combination of the three enzymes and the optimization of the process resulted in a 76 % yield, substantially higher than that obtained by other authors in similar processes (Kanu et al., 2009; Klompong et al., 2012; Romero García et al., 2012). The chemical characterization of the hydrolysate revealed that its amino acid distribution was complete. When comparing the amino acid profile obtained with the yeast extract profile, which is the most widely used nitrogen source in culture media, the hydrolysate was found to have a wider spectrum. This is a very positive factor since there are microorganisms that are not capable of synthesizing certain types of amino acids. By contrast, the amount of total free amino acids in the hydrolysate was less (1.90g/100 g) than that of the yeast extract (3.52 g/100 g).

The range of products that can be obtained via fermentation processes is very wide and varied. This research focused on lactic acid (LA), because it is a compound widely used by many industries, such as the food, chemical, pharmaceutical and cosmetic. Furthermore, its production in recent years has increased considerably due to the growing demand from the plastics industry, which uses it as a precursor to polylactic acid (PLA), a biodegradable polymer with interesting applications

(Komesu et al., 2017). LA can be obtained chemically or biotechnologically. Chemical synthesis always results in the racemic mixture of both isomers, while biological synthesis can form one of the isomers or the mixture, depending on the culture conditions, the type of microorganism and the substrate used. The possibility of producing only one isomer is the main advantage of fermentative production, since in most applications optically pure LA is used. On the other hand, the principal disadvantage is the high cost of substrates (nitrogen and carbon) and downstream processing (extraction and purification). As explained before, the strategy used in this study was to use the nitrogen present in the compost as nitrogen source to obtain LA.

The fermentative production of LA requires the use of microorganisms. In this respect, lactic acid bacteria (LAB) and some filamentous fungi are the main microbial sources of LA. LAB can be classified in two groups, according to the pathway used to metabolize sugars: homofermentative species, transforming sugars mainly into lactic acid through the Embden-Meyerhof-Parnas pathway, and heterofermentative species, transforming sugars into lactic acid, acetic acid, ethanol and carbon dioxide through the phosphoketolase pathway (König et al., 2017).

Within the LAB we found the genus *Lactobacillus*. Most *Lactobacillus* species are highly specialized and are only found in a limited number of places. Contrastingly, others such as *Lactobacillus plantarum* or *Lactobacillus fermentum* are very versatile and widely distributed in most ecosystems, including the gastrointestinal tract of some animals. Both bacteria show high metabolic flexibility such as the ability to metabolize several monosaccharides (glucose, xylose, arabinose, galactose, etc.) simultaneously (Behera et al., 2018; Moura et al., 2007). This is a great advantage, since it would make it possible to combine the hydrolysate rich in amino acids, obtained in this study, with other concentrates rich in sugars, obtained from other residues. Taking these aspects into account, in this study, we decided to use *the L. fermentum* ATCC 9338 and *L. Plantarum* NCIMB 8826 to carry out the fermentative production of LA, despite these being heterofermentative bacteria and producing the mixture of isomers.

As mentioned above, the OFMSW compost hydrolysate contained a comprehensive distribution of amino-acids. This is a positive factor, since many studies have revealed that the LAB have a limited ability to synthesize certain amino-acids and vitamins (König et al., 2017). Thus, the use of the hydrolysate as a source of amino acids was sufficient to cover the nutritional requirements, at least in terms of amino acids, of *L. fermentum* ATCC 9338 and *L. Plantarum* NCIMB 8826.

Many studies have revealed that for a heterofermentative bacterium, if the glucose is metabolized through this metabolic phosphoketolase pathway, only 0.5 g of lactic acid should be produced from 1 g of glucose (0.5 $g_{LA} \cdot g_{glucose}^{-1}$) (Klotz et al., 2016; Lorenzo and Androsch, 2018; Toptas et al., 2014). However, the LA obtained with the selected strains was higher (0.59 $g_{LA} \cdot g_{glucose}^{-1}$, *L. fermentum*; 0.64 $g_{LA} \cdot g_{glucose}^{-1}$ *L. plantarum*). Other authors such as Okano et al. (2009) and Zhang et al. (2016) also obtained higher yields, 0.85 $g_{LA} \cdot g_{glucose}^{-1}$ and 0.77 $g_{LA} \cdot g_{glucose}^{-1}$ respectively, using heterofermentative bacteria. This effect could indicate that the monosaccharides were not only metabolized through phosphoketolase pathway, but also through the Embden-Meyerhof-Parnas pathway, and this is typical of facultative heterofermentative bacteria. In fact, both *L. fermentum* and *L. plantarum* are that type of bacteria (Årsköld et al., 2008; Ascone et al., 2017).

OFMSW compost hydrolysate turned out to be an excellent source of amino acids, but the lack of other important nutrients, namely microelements, vitamins, growth factors, among others, caused a decrease in both cell growth and LA production. This decrease was probably due to the high nutritional requirements of LAB for their development. Nevertheless, it is likely that the lactic acid concentration will substantially increase when production is scaled-up to a bioreactor with sufficient control of process parameters, a more balanced medium and higher glucose concentrations.

It is worth emphasizing that the process developed in this study not only would reduce LA production costs, but also aid in avoiding OFMSW compost overproduction and accumulation problems.

THESIS AND CONCLUSIONS

TESIS Y CONCLUSIONES

THESIS AND CONCLUSIONS

Thesis

Using both OFMSW and its derived compost to produce high-value compounds is a strategy that falls within a circular bioeconomy, and can be combined with existing waste management methods to reduce the waste stream that is disposed of in landfills. However, the high heterogeneity and complexity of the waste represents a disadvantage for its profitability.

Conclusions

Study 1. Assessing the organic fraction of municipal solid waste as carbon source for P(3HB) production.

1. The tandem of thermo-chemical pretreatment and enzymatic hydrolysis with Pentopan 500 BG, Celluclast BG and Glucoamylase NS 22035 turned out to be effective in releasing a large portion of the sugars present in OFMSW.
2. The sugar-rich concentrate, obtained after enzymatic hydrolysis from the OFMSW, proved to be appropriate for the cultivation of *Burkholderia sacchari* DSM 17165.
3. Enrichment of the hydrolysate with salts and adding a pulse of glucose was a good approach to improve the cell growth and P(3HB) production.

Study 2. Scaling-up of poly (3-hydroxybutyrate) production by fed-batch culture of *Burkholderia sacchari* from municipal solid waste hydrolysates and plum waste juices.

4. A combination of OFMSW hydrolysate, as initial culture medium, with a plum concentrate, rich in sugars, turned out to be an excellent strategy for the production of P(3HB) on a laboratory scale.
5. The techno-economic analysis indicated that, under our experimental conditions, the production of a single compound (P(3HB)) from OFMSW and

plum waste juice was not economically feasible due to low productivity and heterogeneity of the carbon source.

6. The highest environmental impacts detected in the P(3HB) production process were related to the use of the extraction solvent (anisole) and to energy consumption.

Study 3. Assessing the organic fraction of municipal solid waste compost as nitrogen source for lactic acid production.

7. The combined use of the endoprotease Alcalase 1.5 MG with the exoprotease Flavourzyme 500 MG released most of the amino acids from the OFMSW compost.
8. The use of the glucanase Viscozyme L in the enzymatic hydrolysis was essential to reduce the viscosity of the medium and increase amino-acid release.
9. The hydrolysate of compost by itself was not enough to satisfy the nutritional requirements of *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB 8826
10. OFMSW compost hydrolysate supplemented with salts was an appropriate medium for the fermentative production of lactic acid by *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB 8826.

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ANNEX

ANEXO

ANNEX 1: PUBLISHED WORKS

MANUSCRIPT 1: Upgrading the organic fraction of municipal solid waste to poly(3-hydroxybutyrate)

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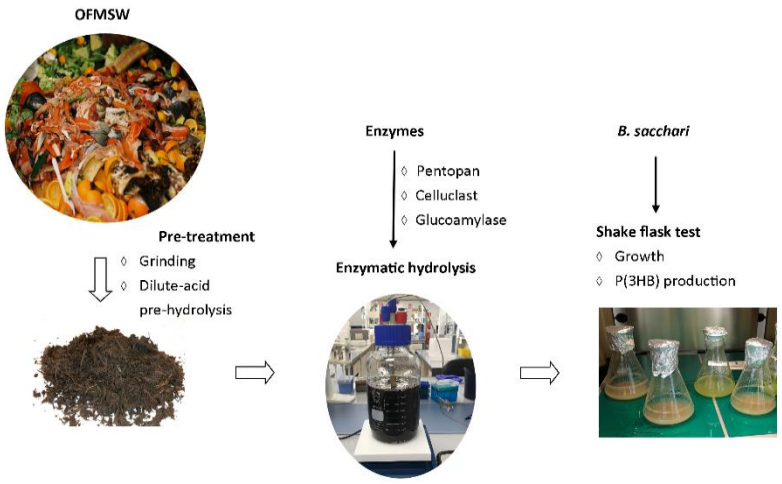
Highlights

- A remarkable release of sugars (56 %) was achieved upon hydrolysis of the OFMSW.
- The OFMSW is an appealing source of nutrients for the growth of *B. sacchari*.
- An increase of the C/N ratio was necessary to promote P(3HB) production.
- The culture accumulated 4.9 g P(3HB) per litre of the supplemented hydrolysate.
- The outlined process adds value to MSW and promotes circular economy.

Abstract

The organic fraction of municipal solid waste was studied as feedstock for the production of poly(3-hydroxybutyrate) (P(3HB)). To release the monosaccharides, a diluted acid pre-treatment followed by an enzymatic hydrolysis was applied. A sugar yield of 49 % was achieved using a pre-treated waste and an enzyme cocktail of Pentopan 500 BG and Celluclast BG. The addition of Glucoamylase NS 22035 helped to hydrolyze the starch fraction, improving the hydrolysis yield to 56 %. The hydrolysate was used as culture medium to produce P(3HB) by *Burkholderia sacchari* DSM 17165. Assays at shaking flask scale showed that when the hydrolysate was used as substrate, the attained cell concentration was slightly higher than in the control medium. It was necessary to supplement the hydrolysate with extra glucose to increase the C/N ratio and with a mineral solution to overcome the nutritional deficiencies. The P(3HB) accumulation using the supplemented hydrolysate was 58 % (g polymer/g biomass).

Graphical abstract



Keywords

Municipal solid waste; Diluted acid pre-treatment; Enzymatic hydrolysis; *Burkholderia sacchari*; Poly(3-hydroxybutyrate)

1. Introduction

Nowadays, plastic has become one of the most indispensable materials in our society. Its great versatility, as well as the low production cost, makes its consumption increase every year. After use, a large fraction of these plastics accumulates in the ecosystems causing serious problems (Geyer et al., 2017). The most efficient way of management is plastic recycling. However, alternative solutions, like the use of biodegradable plastics, are being currently sought (Iwata, 2015; Lambert and Wagner, 2017; Narancic and O'Connor, 2019). Bioplastics are already used in different areas such as agriculture, medicine and packaging, but still represent a small share in the global plastic market. Bio-plastics have a strong potential to become good substitutes for the petroleum-derived plastics due to their adjustable mechanical and thermal properties, as well as for their biodegradability (Brodin et al., 2017; Vigneswari et al., 2009; Muhammad Shamsuddin, 2017). The

most commonly used bioplastics are polylactic acid (PLA), poly(butylene succinate) (PBS), starch based plastics and polyhydroxyalkanotes (PHAs) (Mathuriya and Yakhmi, 2017; Mostafa et al., 2018). Among all types of bioplastics, PHAs attracted the most attention because they are 100 % biodegradable and are produced by a large number of microorganisms. PHAs are composed by hydroxyalkanoic acid monomers bound to each other, forming linear polyesters. Polymer molecular weight (50-1000 kDa) and composition vary with the microbial cultivation mode and conditions, the microorganism and the downstream process (Mathuriya and Yakhmi, 2017). The homopolymer poly-3-hydroxybutyrate (P(3HB)) is the most common type of PHA produced by bacterial cells. The production is growth-associated in some species, while in many others it occurs under unbalanced growth conditions, namely under an excess of carbon and a simultaneous limitation of nutrients such as nitrogen, phosphorous, sulphur, magnesium or oxygen. P(3HB) is accumulated in the cytoplasm in the form of granules, as an intracellular carbon and energy storage compound (Jiang et al., 2015; Alves et al., 2017).

The high production cost of PHAs compared to conventional plastics is the limiting factor for their commercialization at large scale. The price of the carbon source contributes up to 30 % of the total production cost (Plácido et al., 2017; Rodriguez-Perez et al., 2018). Within a circular economy approach, alternatives such as agro-industrial wastes including agricultural residues, forest and garden waste, as well as food and paper waste, can be upgraded to provide sustainable and inexpensive carbon sources (De Corato et al., 2018; Rabaçal et al., 2017; Al-Kindi et al., 2018; Tsang et al., 2019; Valentino et al., 2017; Dietrich et al., 2019). However, it is worthy to note that the wastes commonly used as carbon source at large scale (sugar cane bagasse, corn syrup, rice and wheat straw) are not always available close to the PHA production plants. Waste transportation will thus contribute to the global pollution (Shahzad et al., 2013). The municipal solid waste (MSW) is generated at huge amounts in all cities and its organic fraction (OF) is mainly formed by biodegradable materials (sugars, cellulose, hemicellulose, starch, protein) and

minerals (Campuzano and González-Martínez, 2016). This type of waste has a great potential as sugar platform due to its high availability and carbon content.

Usually, organic wastes start to decompose even before their collection. To prevent the development of microorganisms and maximize the yield of sugars from the organic fraction of MSW, sterilization of the biomass is crucial. Also, preparation of the biomass for the enzymatic hydrolysis involves frequently a pre-treatment step (Karthikeyan et al., 2017) using acidic thermo-chemical hydrolysis. In fact, a pre-treatment followed by an enzymatic hydrolysis is a largely used route to obtain monomeric sugars from lignocellulosic and cellulosic materials (Pleissner et al., 2016; Mahmoodi et al., 2018). Sterilization and pre-treatment of the biomass with diluted acids can thus be combined in a single operation. The use of diluted acids as catalysts reduces the formation of inhibitory compounds (Li et al., 2012), and prepares the biomass for the enzymatic treatment.

The polysaccharide heterogeneity of the organic fraction of municipal solid waste (OFMSW) results in a large range of sugars upon hydrolysis. Hexoses such as glucose, galactose and mannose, are readily consumed by most microorganisms during the fermentation, but not all microbial species are able to consume pentoses (Diaz et al., 2108). For that reason, the selection of a strain able to consume both families of monosaccharides is particularly important to obtain high yields.

The aim of this work was to assess the production of P(3HB) using OFMSW as carbon source. In order to enhance the yield of the hydrolysis process, biomass pre-treatment was evaluated first and then the enzymatic hydrolysis was optimized. The obtained hydrolysate was tested as substrate for P(3HB) production by *Burkholderia sacchari* DSM 17165. This strain is able to grow on various monosaccharides, namely on xylose, and has a remarkable PHA accumulation ability (Bohn et al., 2014; Cesário et al., 2014).

2. Materials and methods

2.1. OFMSW biomass and enzymes

OFMSW was collected in November 2017 from the local composting plant EPELE (Gipuzkoa, Spain). The waste feedstock was composed of pre-screened domestic and garden wastes. After removing the residual impurities (e.g., small stones, plastics, glass and metals), the waste material was lyophilised and milled using a coffee grinder (Moulinex AR100). The final product was packaged in vacuum and stored at -20 °C until its use.

The enzymes used to carry out the enzymatic process were Pentopan 500 BG (xilanase (endo-1,4-)) from *Thermomyces lanuginosus*, (2700 FXU·g^{-1}), Celluclast BG (cellulase from *Trichoderma reesei*, (3500 EGU·g^{-1})) and Glucoamylase NS 22035 from *Aspergillus niger* (750 AGU·g^{-1}), all were supplied by Novozymes S.A. (Madrid, Spain).

2.2. Dilute acid pre-treatment

Prior to the enzymatic hydrolysis a thermo-chemical pre-treatment was carried out. Aiming at this, 0.18 mol·L^{-1} H_2SO_4 was added to OFMSW to attain a biomass load of 135 g·L^{-1} (13.5 %; w/v) and this slurry was heated at 121 °C during 60 min (Pleissner et al., 2016). The pre-treated suspension was cooled and the pH adjusted manually to 4.8 by adding 2 mol·L^{-1} KOH. Finally, the pre-treated biomass was used as substrate for the enzymatic hydrolysis step.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis assays were performed in 250 mL conical flasks using a rotary shaker (Infors HT, Ecotron) for the agitation and temperature control. In this step, the thermo-chemically pre-treated biomass was hydrolyzed using an enzyme cocktail of Pentopan 500 BG, Celluclast BG and Glucoamylase NS 22035. The enzyme load was 90 mg·g^{-1} substrate of Pentopan 500 BG, 150 mg·g^{-1}substrate of Celluclast BG and 108 mg·g^{-1} substrate of Glucoamylase NS 22035. The reaction was conducted

at pH 4.8, 170 rpm and 50 °C during 48 h. To evaluate the amount of sugar released throughout the hydrolysis, 1 mL samples were taken at different times. At the end of the hydrolysis the sugar-rich supernatant was separated from the pellet by centrifugation at 10,000 rpm (Beckman Coulter Avanti J-26 XP) and the final concentrate clarified using an 11 µm paper filter (Whatman). Finally, the sugar concentrate was sterilized at 121 °C for 20 minutes. To determine the influence of the biomass load, enzymatic assays were performed at different biomass concentrations (50-170 $g \cdot L^{-1}$) maintaining the hydrolysis conditions as described above. To produce larger volumes of the OFMSW hydrolysate, a 2 L stirred reactor (Duran GLS 80) was used under the same conditions mentioned above. To maintain the temperature, it was necessary to introduce the reactor in an oven (Memmert INB200).

The yield of hydrolysis (equation (1)) was estimated as the ratio between the concentration of the released sugars (glucose, xylose and arabinose) and the polysaccharides concentration (calculated as a fraction of the biomass concentration). The 0.9 factor corresponds to the correction that should be made when considering the hydrolysis of an anhydrous polysaccharide form into its monosaccharides [0.9 = glucose in polymeric glucan (MW = 162) / monomeric glucose (MW = 180)]. The same factor was used for the hydrolysis of the hemicellulose and starch (Pleissner et al., 2016).

$$\textbf{Hydrolysis degree}\ (\%) = \frac{\textbf{Released sugars}\ (g \cdot L^{-1}) \times 0.9}{\textbf{Cellulose}\ (g \cdot L^{-1}) + \textbf{hemicellulose}\ (g \cdot L^{-1}) + \textbf{starch}\ (g \cdot L^{-1})} \times 100 \quad (1)$$

2.4. Microorganisms, culture media and inocula preparation

Burkholderia sacchari DSM 17165 was maintained in Luria Bertani broth (LB) agar stabs and stored at 4 °C. A culture bank was prepared in cryovials and stored at -80 °C. For this, 1500 µL of a culture in the late exponential phase was frozen in cryogenic tubes containing 300 mL of glycerol.

The basal medium consisted of a simple salts medium containing the following in g/L: $(NH_4)_2SO_4$, 1.0; $Na_2HPO_4 \cdot 2H_2O$, 4.5; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.2; yeast

extract, 1.0 and 1.0 mL of trace elements solution, the composition of which per liter was: $FeS0_4 \cdot 7H_20$, 10 g; $ZnS0_4 \cdot 7H_20$, 2.25 g; $CuSO_4 \cdot 5H_20$, 1 g; $MnS0_4 \cdot 5H_20$, 0.5 g; $CaCl_2 \cdot 2H_20$, 2 g; $Na_2B40_7 \cdot 10H_20$, 0.23 g; $(NH_4)_6Mo_70_{24}$, 0.1 g; 35 % HC1 10 mL (Cesário et al., 2014). In the described medium, nitrogen was set the limiting nutrient. The sugar and the $MgSO_4 \cdot 7H_2O$ solutions were autoclaved separately and aseptically added to the medium. The pH of the medium was adjusted to 6.8 with KOH.

The inoculum to the shake flask assays was prepared transferring the content of a cryovial to a flask containing 50 mL of the mineral medium and incubated for 12 h at 30 ̊C and 170 rpm in an orbital shaker (Cesário et al., 2014).

2.5. Utilization of OFMSW hydrolysate for PHA production

The shake flask experiments were carried out to assess the value of the OFMSW real hydrolysates as substrate for the P(3HB) production. For that, the growth and the P(3HB) production by *Burkholderia sacchari* DSM 17165 were compared between the hydrolysate and the control medium, i.e., a basal medium with addition of a solution of sugars simulating the sugar composition of the hydrolysate. These assays were performed in 500 mL conical flasks containing 100 mL of control medium or hydrolysate. Flasks were inoculated with 10 % (v/v) of an overnight grown culture and incubated at 30 °C during 70 h at 170 rpm. Samples from each flask were taken at different time intervals to measure optical density (OD 600 nm), cell dry weight (CDW), P(3HB) content and sugars concentration. All experiments were carried out in duplicate.

2.7. Analytical methods

Bacterial growth was evaluated by the spectrometric method based on the determination of optical density (OD) at 600 nm using a spectrophotometer (Shimadzu UV-2401PC). CDW was determined by centrifuging 1.2 mL of culture sample in a Sigma 1-15PK centrifuge (5 min; 14000 rpm) using previously dried and weighed 1.5 mL microtubes. The supernatant (1.0 mL) was stored at -18 °C for the

analysis of sugars. The pellet obtained after sample centrifugation was washed with distilled water (1 mL) and dried in an oven (Selecta 200) at 60 °C until constant weight.

The dry matter of the OFMSW biomass was determined by weighing a certain amount of biomass and drying it in an oven at 105 °C during 24 hours until constant weight.

Sugars present in the culture broth and in the OFMSW hydrolysates were determined by high performance liquid chromatography (HPLC) (Agilent Technologies 1260 Infinity II) equipped with a Hi-Plex H (300 mm x 7.7 mm) column and an auto sampler (Agilent 1260 Vialsampler G7129A). Detection was carried out by a refraction index detector Agilent 1260 RID G7162A. The column work temperature was 65 °C. The injection volume was 2.4 µL and as eluent a 5 mM H_2SO_4 solution was used with a flow rate of 0.5 mL/min. Each analysis was carried out in duplicate and peak identification and quantification was achieved using standards of glucose, xylose and arabinose.

P(3HB) identification and quantification was carried out by Gas Chromatography - Mass Spectrometry (GC-MS). For that, 1.2 mL of samples were taken from the culture broth. The pellet obtained by centrifugation was methylated following the procedure described in a previous study (Cesário et al., 2014). Monomers were analysed using a 7890 A gas chromatograph (Agilent Technologies, Avondale, PA, USA) coupled to a 5975 C electron impact ionization mass spectrometer and 7683 B Agilent autosampler. The analyses were performed in the splitless mode for 2 min at 120 °C into a HP-5MS UI capillary column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies, Avondale, PA, USA). The oven temperature program used was programmed from 40 °C to 60 °C at 10 °C·min^{-1} (hold 4 min) and then until 230 °C at 50 °C·min^{-1} where it was maintained for 1 min. Helium was used as carrier gas at a constant flow of 1 mL·min^{-1}. The MS transfer line temperature was maintained at 280 °C and the ion source and quadrupole at 230 °C and 150 °C, respectively.

Measurements were performed in SCAN (40-600 m/z) mode. The calibration curves were made using a 3-methyl hydroxybutyrate standard (Sigma Aldrich).

Fiber analysis (cellulose, hemicellulose and lignin) analyses were carried out using an ANKOM 2000 fiber analyzer (Komarek et al., 1996).

Total carbon, nitrogen and hydrogen were determined using an elemental analyzer (Leco CHNS-932). The protein content (%) in the OFMSW biomass was calculated as 6.25 times the total percent nitrogen in the sample (AOAC, 1990). The fats were extracted according to the Folch, Less and Stanley method (1957) and determined by gravimetry.

2.8. Statistical analysis

Results are presented as means value ± standard deviation. To see the differences between the means of each group one-way analysis of variance (ANOVA) and 95 % confidence levels were applied. Data with P-value < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Chemical characterization of OFMSW

The OFMSW has a high heterogeneity and complexity. Its composition depends on many factors, namely on the geographical location, the people´s culture, education and the climate (Mahmoodi et al., 2018). A further strong influencing factor is the policy of the municipality for the encouragement and implementation of separate waste collection. In the present case a mixture of domestic and garden waste was addressed. It was thus to be expected that the OFMSW would contain complex carbon compounds. In fact, the compositional analysis showed that the pre-screened material was rich in fiber (47 % w/w), which can potentially be transformed to utilizable monomers by microorganisms for the production of polyhydroxyalkanoates. Besides, the analysis also revealed that it contained appreciable quantities of ash (34 %; w/w) proteins (10 %; w/w), reducing sugars (3

%; w/w), fats (2 %; w/w), starch (2 %; w/w) and others (2 %; w/w). A similar composition of OFMSW was obtained by Ghanavati et al. (2015).

3.2. Diluted acid pre-treatment

As mentioned above, the waste used in this work contained a remarkable amount of fiber (47 %; w/w) composed predominantly of cellulose, hemicellulose and lignin. The structural properties of this type of biomass make the access of the enzymes rather difficult. To improve the yield of the saccharification process, a pre-treatment of the biomass was carried out. This was attained by a thermo-chemical process with the aid of an acid catalyst. The application of mild conditions, i.e. the use of diluted acid is recommended to prevent the release of toxic compounds (e.g. furfural and hydroxymethyl furfural) when aiming to use the hydrolysate in subsequent fermentative processes (Sørensen et al., 2008). Parameters such as duration of the treatment and temperature also influence the final sugar concentration (Vavouraki et al., 2013). With this particular waste, a 60 minute pre-treatment was necessary to simultaneously sterilize the biomass. This is required due to the high concentration of microorganisms in the OFMSW that would otherwise affect the final sugar concentration in the hydrolysates. The temperature of 121 °C was selected (Gonzales et al., 2016; Kim et al., 2018).

Finally, the thermo-chemical pre-treatment of OFMSW was carried out prior to the enzymatic hydrolysis during 60 min at 121 °C in presence of 0.18 $mol \cdot L^{-1}$ H_2SO_4. The results showed an improvement in the degree of hydrolysis after the enzymatic hydrolysis of 28 % (Figure 1).

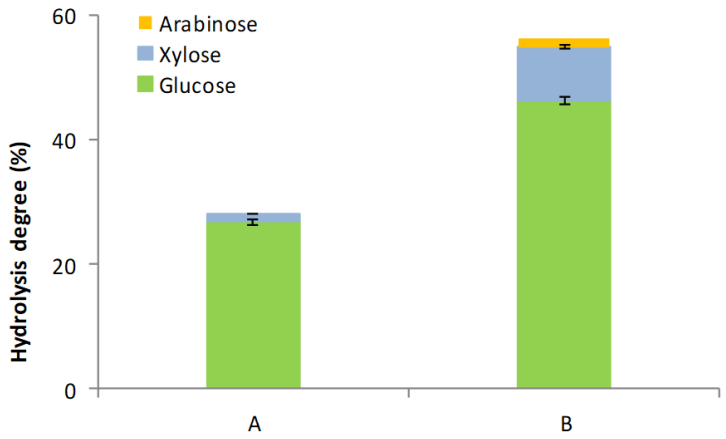


Fig. 1*:* Released sugars (%) after enzymatic hydrolysis, without (A) and with pre-treatment (B).

3.3. Enzymatic hydrolysis

The high amount of cellulose, hemicellulose and lignin-like polysaccharides in the waste required a careful selection of the enzyme cocktail. To carry out an effective hydrolysis the synergistic action of a mix of enzymes was necessary. In this work two enzyme preparations, Pentopan 500 BG and a Celluclast BG were used. Pentopan 500 BG is a xylanase, which cleaves internal β-1,4-glucosidic bonds of xylanes. On the other hand, Celluclast BG comprises three types of enzymes: an endoglucanase, which hydrolyses accessible β-1,4-glucosidic bonds; an exoglucanase, which releases short-chain cellobiose units; and finally, a β-glucosidase, which hydrolyses cello-oligomers to glucose.

To achieve a high degree of hydrolysis, besides enzyme selection, hydrolysis duration and enzyme:substrate ratio were considered. The first analysed parameter was the reaction time. To determine its influence in the hydrolytic process, the enzymatic hydrolysis of pre-treated biomass was followed during 60 h (Figure 2). The glucose and xylose release was very fast in the first ten hours, but then the increment slowed down. A long hydrolysis renders the process too expensive, while

a shorter treatment does not reach an adequate sugar conversion yield. As a compromise, the duration of the enzymatic process was established at 48 h.

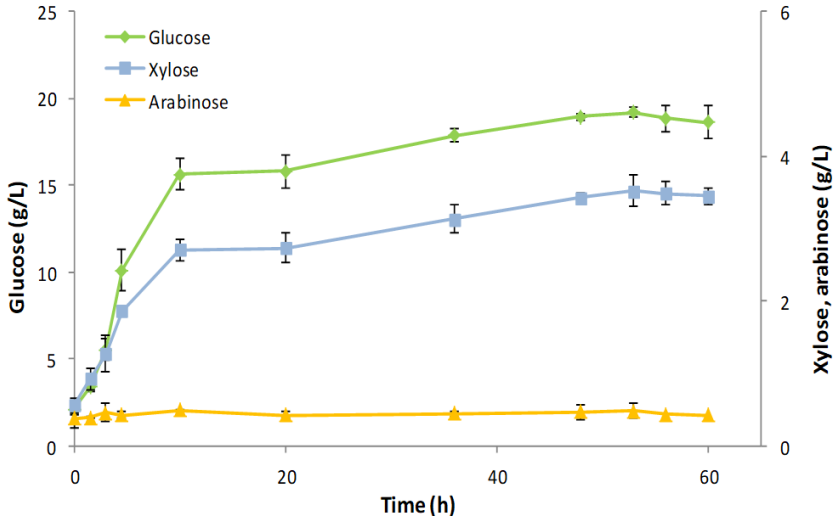


Fig. 2: Release of sugars during the enzymatic hydrolysis using 90 mg of Pentopan 500 BG per gram substrate and 150 mg of of Celluclast BG per gram of substrate; pH 4.8; temperature 50 °C.

Next, the influence of the enzyme:substrate ratio was investigated. In the first assay a fixed substrate concentration of 135 g·L^{-1} and a low enzyme load (9 mg Pentopan·g^{-1} substrate; 15 mg Celluclast ·g^{-1} substrate) were used. Then, the enzyme concentration was increased two, five, ten and twenty times (X2, X5, X10 and X20 in Figure 3) to attain a maximum hydrolysis degree of 49 % with an enzyme load 20 times higher than the initial load. Considering the reduced gain in the degree of hydrolysis between X10 and X20 and the enzyme cost, the use of Pentopan 500 BG and Celluclast BG at 90 mg·g-1 substrate and 150 mg·g-1 substrate respectively, was taken as adequate.

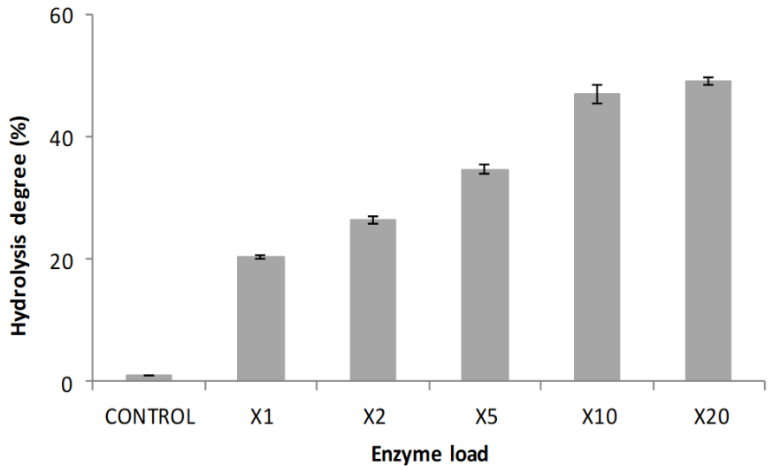


Fig. 3: Effect of the enzyme load (X1: 9 mg·g^{-1} substrate Pentopan 500 BG and 15 mg·g^{-1} substrate Celluclast BG; 48h; pH 4.8; 50 °C) on the hydrolysis degree. X2, X5, X10 and X20 corresponded to the different enzyme loads used and were two, five, ten and twenty times higher than X1, respectively. Control had not enzyme addition.

The effect of the biomass concentration on the enzymatic hydrolysis was also determined. This study can be approached by maintaining constant the biomass and modifying the water volume or vice versa. In this work the first scenario was used and the best enzyme/substrate ratio previously determined was maintained. For that, experiments using the same biomass and enzyme quantity and varying the water volume were carried out. The biomass concentrations studied were in the range 50 g·L^{-1} to 170 g·L^{-1}. The results showed that, the sugar concentration was enhanced with the increase in biomass concentration up to a certain biomass concentration value. However, higher solid loads usually generate a high viscosity, resulting in lower yields (Vani et al., 2015). The maximum sugar concentration was achieved with 135 g·L^{-1} substrate, but this did not correspond to the highest hydrolysis degree, which was attained with a biomass of 90 g·L^{-1} (Figure 4). Despite a lower hydrolysis degree, a biomass load of 135 g·L^{-1} was selected to produce sugar-rich hydrolysates for the bacterial cultivation. Under these conditions 19.0 g·L^{-1}, 3.4 g·L^{-1} and 0.50 g·L^{-1} of glucose, xylose and arabinose, respectively, were attained.

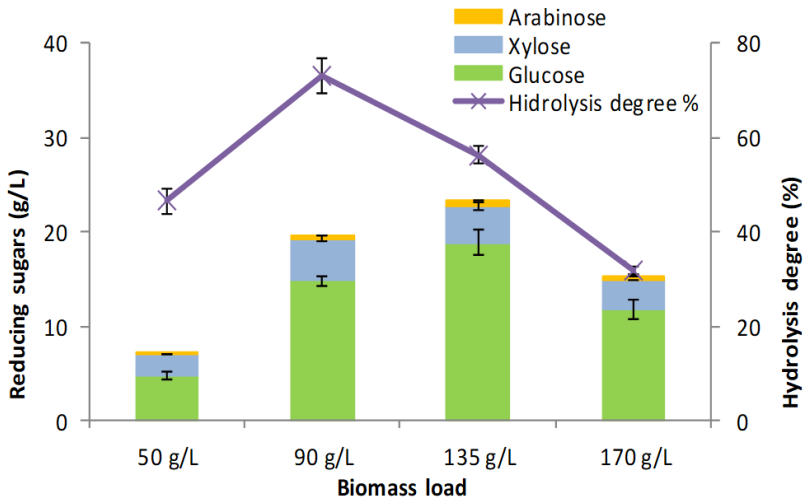


Fig. 4: Influence of biomass concentration on the hydrolysis degree and released sugars after enzymatic hydrolysis using 90 mg Pentopan 500 BG and 150 mg Celluclast BG per gram of substrate; time 48h; pH 4.8; temperature 50 °C.

The chemical characterization of OFMSW showed that it contained a small starch fraction (2 %; w/w). To improve the sugars concentration in the final product, Glucoamylase NS 2235 was added to the enzyme cocktail at a concentration of 108 $mg \cdot g^{-1}$ substrate. The results showed that, with a biomass concentration of 135 g/L, the addition of glucoamylase to the enzyme cocktail increased the sugar yield from 49 % to 56 %. Similar yields using municipal solid waste as substrate in analogous processes were obtained by (Li et al., 2012) and by (Li et al., 2007).

3.4. Chemical composition of OFMSW hydrolysate

Table 1 summarizes the chemical composition of the hydrolysate obtained from OFMSW upon enzymatic hydrolysis using 90 $mg \cdot g^{-1}$ substrate of Pentopan 500 BG, 150 $mg \cdot g^{-1}$ substrate of Celluclast BG and 108 $mg \cdot g^{-1}$ substrate of Glucoamylase NS 22035, at 50 ̊C and pH 4.8 for 48 h. Glucose was the main monosaccharide, although the pentoses, xylose and arabinose, were also present due to the hemicellulosic fraction. The most abundant elements in the hydrolysate were nitrogen, calcium, phosphorus, magnesium and iron, which are essential components of the basal culture media. Heavy metals were present in low concentrations and did not

represent any problem during the growth tests. The high nutritious value, as well as the low quantity of heavy metals, suggested the possibility of using the hydrolysate as culture medium without adding extra nutrients.

Table 1: Composition of the OFMSW enzymatic hydrolysate.

Parameter	Unit	Concentration
Dry weight	%	7.3
Ash	%	41.5
Glucose	g/L	20.8
Xylose	g/L	3.9
Arabinose	g/L	0.6
N	g/L	1.5
P	mg/L	150.8
Ca	mg/L	790.2
Mg	mg/L	120.5
Cu	mg/L	0.02
Cr	mg/L	1.3
Fe	mg/L	113.9
Mn	mg/L	9.9
Mo	mg/L	0.1
Ni	mg/L	0.2
Zn	mg/L	1.3
As	mg/L	0.1

3.5. Growth and P(3HB) production tests using the OFMSW hydrolysate

As shown above, glucose was the dominant sugar in the OFMSW hydrolysate. This is a positive factor, since the metabolization of hexoses provides more energy to the cell, and therefore might produce a higher P(3HB) amount (Obruca et al, 2014). Cesário et al., 2014 used *Burkholderia sacchari* DSM 17165 to produce P(3HB) from a wheat straw hydrolysate with a high pentose content. Due to the

similarity of the type of sugars released, the same strain was selected to carry out this work.

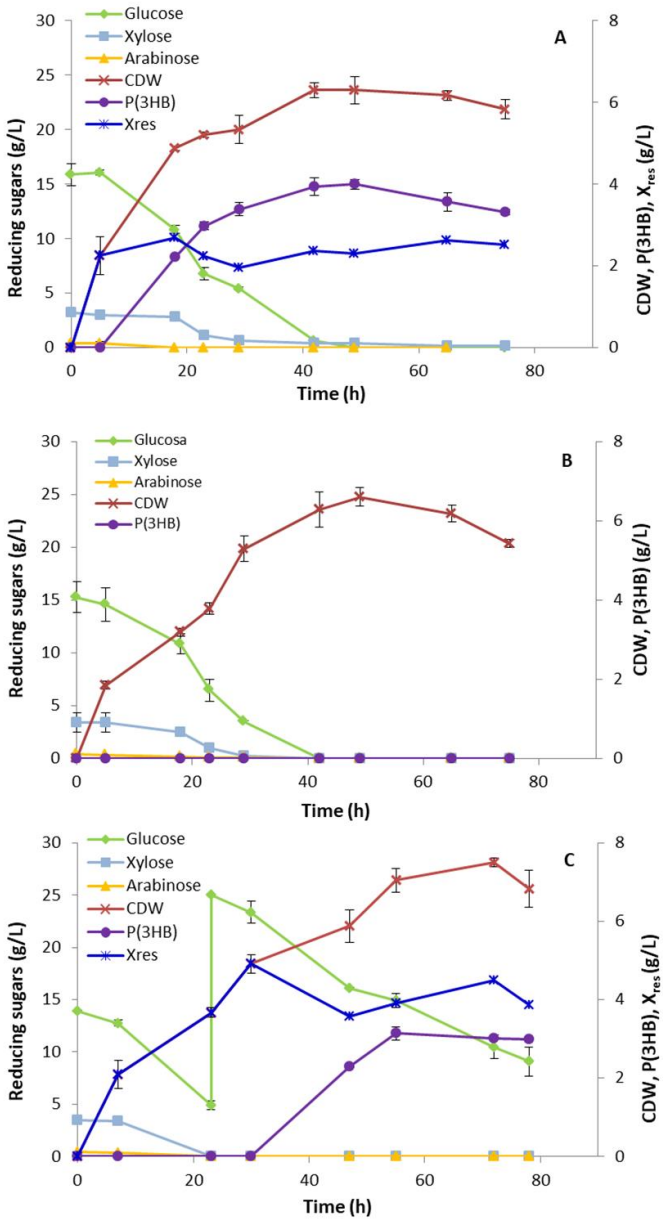


Fig. 5: Growth and P(3HB) production of *B. sacchari* on A) control medium, B) OFMSW hydrolysate and C) OFMSW hydrolysate with a glucose pulse at 23 h of cultivation time.

In order to determine whether the OFMSW hydrolysate was adequate for P(3HB) production, shaking flask assays were performed. Initially, the cell growth and polymer production were studied using mixtures of glucose, xylose and arabinose in the same concentrations as in the hydrolysate (Figure 5A) and were compared with the growth and production obtained on the OFMSW hydrolysate. Although the results indicated similar growth, P(3HB) production was only achieved with the sugar mixture control medium (Figure 5B). This fact could be ascribed to the low carbon/nitrogen ratio (7.4) at the beginning of the assay performed with the hydrolysate. Some authors have described that it is necessary to have C/N ratios above 20 to obtain a significant polymer accumulation (Singh Saharan et al., 2014; Lopes et al., 2014). Therefore, in order to induce P(3HB) production on the hydrolysate, a further assay was carried out where a pulse of 20 $g \cdot L^{-1}$ of glucose was added at 23h of cultivation time, before glucose was exhausted in the medium (Figure 5C). Nitrogen and carbon were consumed in the first 23 h promoting solely cell growth to approximately 4 g/L CDW. After addition of a glucose pulse to the medium, the C/N ratio increased and from that moment on the use of carbon was mainly destined for polymer production. At large scale, the C/N ratio could be enhanced by the addition of other residues with high-carbon content, namely fruit waste concentrates.

Table 2 Growth and P(3HB) production parameters of *Burkholderia sacchari* in shake flask cultures carried out with OFMSW pre-treated enzymatic hydrolysate and hydrolysate supplemented with salts as compared to a control with commercial sugars.

Assay medium	**Consumed sugars (g/L)**	**CDW_{max} (g/L)**	**$P(3HB)_{max}$ (g/L)**	**$Y_{p/s}$ ($g_{P(3HB)}/g_{sugar}$)**	**P(3HB) (%)**	**Time (h)**	**$Prod_{vol}$ ($g_{P(3HB)}$ /(L·h)**
Control	19.4 ± 1.9	6.00 ± 0.98	3.90 ± 0.16	0.20 ± 0.04	63.0 ± 2.9	42	0.09 ± 0.01
Hydrolysate	28.9 ± 2.3	7.50 ± 1.11	3.11 ± 0.11	0.11 ± 0.06	44.0 ± 2.3	55	0.06 ± 0,01
Hydrolysate + salts	38.1 ± 1.1	8.80 ± 0.69	4.92 ± 0.31	0.13 ± 0.02	58.0 ± 1.9	73	0.07 ± 0.03

The yield of polymer on sugar consumed, $Y_{P/S}$ (0.20 g $_{P(3HB)}$/g $_{sugar}$) and the volumetric productivity, $Prod_{vol}$ (0.09 $g_{P(3HB)}$ $\cdot L^{-1} \cdot h^{-1}$) were higher on the control medium compared to the results attained on the hydrolysate supplemented with glucose. In the latter, a higher residual biomass was produced instead (X_{res} = 4.39 $g \cdot L^{-1}$ compared to the control medium X_{res}= 2.10 $g \cdot L^{-1}$). As referred above this may be ascribed to the initial low C/N ratio of the hydrolysate. The results are summarized in Table 2.

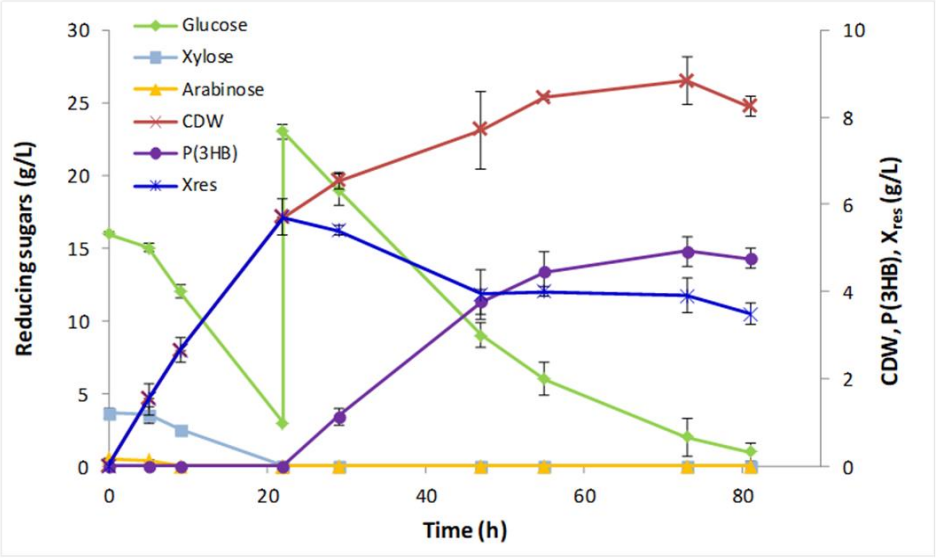


Fig. 6: Growth and P(3HB) production of *B. sacchari* on OFMSW hydrolysate supplemented with salts.

Figure 5C shows that polymer production stopped at approximately 55 h cultivation time, although glucose was not exhausted at this point. This might indicate the lack of some other nutrient in the medium (M. Kellerhals et al., 1999; Koller., 2017). To ascertain whether this was the case, another shake flask assay was performed by supplementing the hydrolysate with 10 mL of a 10-fold concentrated basal medium. The composition of this basal medium was similar to the basal medium described above, but lacking $(NH_4)_2SO_4$ and yeast extract so as to maintain the C/N ratio approximately constant. Figure 6 shows that upon the

supplementation of basal medium, both cell dry weight (8.8 $g \cdot L^{-1}$) and P(3HB) production (4.92 $g \cdot L^{-1}$) increased. However, this was neither translated in an increase of $Y_{P/S}$ (0.13 $g_{P(3HB)}/g_{sugar}$) nor in an increase of the $Prod_{vol}$ (0.07 $g_{P(3HB)} \cdot L^{-1} \cdot h^{-1}$), which were still lower compared to the control. As before, the polymer production started much later (22 h) and the X_{res} (3.71 $g \cdot L^{-1}$) was higher than on the control medium. This effect can be due to the delay in the P(3HB) production or also because the hydrolysate favours biomass production in detriment of P(3HB) accumulation (Al-Kindi et al., 2019). In spite of the high complexity and heterogeneous nature of the OFMSW, the data obtained are in conformity with the findings of Silva et al. (2004); Al-Kindi et al. (2019) and Cesário et al. (2014), who obtained similar results at shake flask scale (CDW, P(3HB) accumulation and X_{res}, using sugarcane bagasse, waste paper and wheat straw hydrolysates respectively. Besides, the composition of the OFMSW will vary from batch to batch. For that reason, different batches should be tested and the effect on the polymer productivity assessed. Finally, further studies including limiting nutrient selection, process optimization, scale up and economic analysis will have to be performed to ascertain the process feasibility.

In summary, the present study indicates that carbon-rich hydrolysates obtained from the organic fraction of municipal solid waste are a good substrate for P(3HB) production.

4. Conclusions

Both pre-treatment and enzymatic hydrolysis optimization were key steps to obtain a high release of sugars from OFMSW. The obtained hydrolysate was an appealing substrate that satisfied the nutritional requirements of *Burkholderia sacchari*. However, to promote P(3HB) production a glucose pulse was needed to increase the C/N ratio. For scaling-up, high-carbon content residues like fruit waste concentrates could replace glucose addition. Finally, the supplementation of a balanced salts medium improved cell growth and P(3HB) production. The use of

wastes for P(3HB) production decreases the production cost and alleviates the amount of waste in the environment, contributing to a circular economy.

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MANUSCRIPT 2: Giving credit to residual bioresources: from municipal solid waste hydrolysate and waste plum juice to poly(3-hydroxybutyrate)

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Highlights

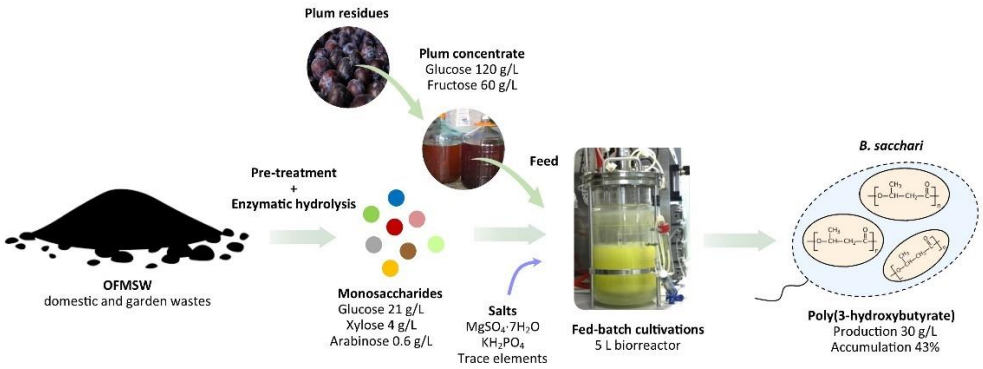
- Previously validated OFMSW hydrolysate was used to lab scale P(3HB) production.
- The OFMSW hydrolysate is a suitable substrate for use as media in the batch phase.
- Sugar rich plum concentrate promotes the production of P(3HB) in fed-batch phase.
- Combining both substrates, the yield was 0.15 g P(3HB) per gram of sugar consumed.
- The suggested process can integrate into a circular economy model.

Abstract

Municipal solid waste (MSW) is massively generated all over the world. Its organic fraction (OFMSW), which represents a high percentage of MSW, mainly contains biodegradable materials, namely food waste, paper and garden waste. The social cost of OFMSW treatment and/or disposal is a serious and widespread problem, particularly in highly populated areas. Thus, effective and innovative solutions, which include the upgrading of OFMSW, are being currently sought. In fact, the OFMSW abundance, availability and average composition suggest its considerable potential within the circular economy desideratum, paving the way to valorisation approaches. In this context, an OFMSW sugar-rich hydrolysate and its validation as a substrate for the production of the polyester poly(3-hydroxybutyrate) (P(3HB)), to date the only bioplastic easily biodegradable in marine environment, were successfully obtained in a previous study. Based on those results, this work addresses the upscaling of the fermentative production, in fed-batch mode, of P(3HB) by *Burkholderia sacchari*. The OFMSW hydrolysate was used as cultivation medium due to its balanced nutrient composition, while a plum waste

juice, also rich in sugars, was applied as feed to the bioreactor. By implementing this strategy, a maximum P(3HB) production of 30 g·L^{-1} with an accumulation of 43 % g (P(3HB))/g cell dry weight (CDW) after 51 h, was achieved. The use of the hydrolysate as initial medium resulted in higher CDW (71 g·L^{-1}) than that of the simulated hydrolysate (62 g·L^{-1} in average), probably because the OFMSW hydrolysate favours biomass growth in detriment of P(3HB) production.

Graphical abstract



Keywords

Municipal solid waste; Bacterial cultivation; Fed-batch; *Burkholderia sacchari*; Poly(3-hydroxybutyrate)

Abbreviation list

CDW, cell dry weight; CDW_{max}, maximum cell dry weight; DO, dissolved oxygen; EU, European Union; GC-MS, gas chromatography-mass spectrometry; MSW, municipal solid waste; OD_{600}, optical density at 600 nm; OFMSW, organic fraction of municipal solid waste; P(3HB), poly(3-hydroxybutyrate); P(3HB) %, Accumulation percentage of poly (3-hydroxybutyrate); $P(3HB)_{max}$, maximum production of poly (3-hydroxybutyrate); PHAs, polyhydroxyalkanoates; PLA, polylactic acid; $Prod_{vol}$, volumetric productivity; X_{res}, residual biomass; $Y_{p/s}$, yield of polymer on sugar consumed

1. Introduction

In the present days it is rare to find an object that does not contain a fraction of a petroleum-derived plastic, however small it may be. The excellent and versatile properties of plastics obtained by the petrochemical industry have led to an exponential increase in the scale of their production over the last decades. According to Lebreton et al. (2017), plastics production worldwide broke the barrier of 300 million tons in 2014 and it is foreseen that it will continue to increase in the forthcoming years. Moreover, their low price and ease of production favour the utilization of single-use plastics. This issue combined with their widespread resilience to biodegradation generates huge environmental concerns (Sardon and Dove, 2018). To date, disposal in land-fills and pyrolysis are the principal ways to manage plastic wastes. The later solutions only hinder the mitigation of the problem (Geyer et al., 2017). Recycling is another alternative, however only 5 % of manufactured plastics are recycled. On the other hand, strategies such as reduction of their use and partial or total replacement by biodegradable polymers (bioplastics) are taking hold in recent years (Prata et al., 2019). The best known biopolymers are the aliphatic polyesters, namely polylactic acid (PLA) and the polyhydroxyalkanoates family (PHAs), polysaccharides (e.g. cellulose and starch) and some polypeptides (e.g. collagen) (Sabbah and Porta, 2017). In particular, PHAs, which are produced as intracellular carbon/energy storage under nutrient limitation (of e.g. nitrogen, phosphorous or oxygen) by a large variety of microorganisms, are attracting much attention because they can be tailored so as to offer similar properties to petroleum-derived polymers and, in addition, are completely biodegradable (Kabir et al., 2017). It is worth to mention that PHAs are the only bioplastics that are degradable in the marine environment (Rujnić-Sokele and Pilipović, 2017; DiGregorio, 2009).

Among the PHA types, poly(3-hydroxybutyrate) (P(3HB)) is the most widely studied and is already used to make a large range of products such as packaging products, bottles and containers, electrical equipment, shopping bags, etc. (Misra et al., 2006; Sudesh et al., 2000; Albuquerque and Malafaia, 2018). However, the

production cost of PHAs is considerably higher as compared to conventional plastics. At present, and as a result, society depends to a large extent on non-biodegradable polymers (Dietrich et al., 2017).

The major expenses in P(3HB) production by bacterial species are associated with the cost of the fermentation substrate, the maintenance of sterile conditions and the polymer extraction. In fact, the cost of the carbon source may account for 30 % to 50 % of the total production cost (de Paula et al., 2017; García et al., 2019; Obruca et al., 2015). Considering these facts, the key to obtain an economic carbon source appears to be the use of sugar rich substrates like e.g. hydrolysates of agricultural and industrial wastes or the organic fraction of municipal solid waste (OFMSW) (Yusuf, 2017; Ghanavati et al., 2015). In reality the OFMSW is one of the most abundant feedstocks in cities, accounting for approximately 30-40 % of municipal solid waste (MSW) in Europe (Eurostat, 2018; Cesaro et al., 2019). Traditionally, most of this waste was deposed in landfills where its decomposition has been related to environmental problems such as gas emissions, leachate of pollutants and animal diseases, among others. Later, alternative forms of management were developed, which were more environmentally friendly, the most important being composting, mechanical-biological treatment, biogas production and incineration (Kumar and Samadder, 2017; Trulli et al., 2018). Nevertheless, these strategies do often generate products with low added value to be considered economically feasible(Clarke, 2018). Nowadays, instead of a nuisance this type of waste is considered a valuable resource with which added-value products can be manufactured. Recently, Izaguirre et al. (2019) reported the production of P(3HB) by *Burkholderia sacchari* DSM 17165 using OFMSW as substrate and other authors used the same substrate to produce lipids, lactic acid, biofuels, volatile fatty acids, etc. (Gameiro et al., 2016; Ghanavati et al., 2015; López-Gómez et al., 2019; Mahmoodi et al., 2018).

The utilization of wastes to obtain new products reduces the environmental contamination, the greenhouse-gas emissions and the use of energy, and, moreover, meets the demands of a circular economy. In the last years this concept is gaining

much attention and thus the European Union (EU) has adopted several initiatives outlined in the Circular Economy Action Plan (EC, 2015). This plan claims an increase in the MSW recycling rate and a reduction in landfilling. Bio-plastics production would not only alleviate the plastics pressure over the environment, but also reduce the amount of waste in landfills. To date and unfortunately these points have not been taken into consideration when the cost of bio-plastics is estimated, although they should be carefully considered when the life cycle analysis of plastics is addressed. According to (Vethaak and Leslie, 2016; Nizami et al., 2017; Guillard et al., 2018), the positive impact of the replacement of petroleum derived plastics by bioplastics would involve huge savings in environmental and health aspects.

This work is based on the preliminary data obtained by Izaguirre et al. (2019), where the production of P(3HB) was assessed at shaking flask scale using OFMSW hydrolysates as carbon source. The aim of the present study was the scaling-up of the process to a 5 L stirred tank reactor operated in the fed-batch-mode. The bacterial cells were grown on OFMSW as the only carbon source and the possibility of using plum concentrates as feed was evaluated. The results obtained are expected to shed light on the valorisation of urban solid wastes and help solve problems related to their management.

2. Materials and methods

2.1. Raw materials

2.1.1. OFMSW

The OFMSW samples were collected in November 2017 from EPELE composting plant, located in Gipuzkoa, Spain. They were formed by pre-screened household and garden wastes, and their treatment and composition has been recently described (Izaguirre et al., 2019).

2.1.2. Plum concentrate

A plum waste concentrate with a sugar composition of 120 g·L^{-1} glucose and 60 g·L^{-1} fructose was kindly provided by a bioresidues upgrader company (CATAR-CRIT Agroressources, France). This juice was obtained by thermo-mechanical extraction of plum wastes and was used as feed in some of the bioreactor fed-batch assays.

2.1.3. OFMSW hydrolysate preparation

To release the sugars from this feedstock a thermo-chemical pre-treatment followed by an enzymatic hydrolysis was carried out, as previously described by Izaguirre et al. (2019). Briefly, a 13.5 % w/v OFMSW suspension was first pre-treated with H_2SO_4 0.18 mol·L^{-1} at 121 °C during 60 min using a 2 L stirred reactor (Duran GLS 80). The obtained suspension was chilled and the pH was raised to 4.8 with a solution of KOH 2 mol·L^{-1}. As a second step, the dilute acid pre-treated OFMSW was enzymatically hydrolysed, using an enzyme mixture of Pentopan 500 BG, Celluclast BG and Glucoamylase NS 22035. The enzyme load per gram of substrate was 90 mg of Pentopan 500 BG, 150 mg of Celluclast BG and 108 mg of Glucoamylase NS 22035. The reaction was completed after 48 h, and the conditions of temperature, pH and agitation were 50 °C, 4.8 and 170 rpm respectively. When the reaction ended, the residual pellet was removed from the sugar-rich solution by centrifugation at 10,000 rpm (Beckman Coulter Avanti J-26 XP), and the supernatant filtered using a 11 µm paper filter (Whatman). The resulting hydrolysate was sterilized in an autoclave (121 °C, 20 min).

Finally, the sugars composition of the hydrolysate was 21 g·L^{-1} glucose, 4 g·L^{-1} xylose and 0.6 g·L^{-1} arabinose.

2.2. Chemicals, microorganism, culture media and inoculum preparation

As described previously by Izaguirre et al. (2019), to preserve the strain *Burkholderia sacchari* DSM 17165, recently reclassified as *Paraburkholderia*

sacchari, under optimal conditions, a culture bank was prepared in cryovials. These were kept at -80 °C until their use as starter inocula.

The medium for the shake flask assays was formed by a simple salt medium as previously described (Izaguirre et al., 2019). Basal medium or OFMSW hydrolysate were used to initiate the fed-batch fermentations. The basal medium consisting of a mixture of commercial sugars with the same composition as the hydrolysate was used as reference medium to validate the results using the hydrolysate for the production of P(3HB). As referred by Cesário et al. (2014), this medium was designed for phosphorus to be the first limiting nutrient. Salts and trace elements were obtained from Sigma-Aldrich (Steinheim, Germany; ≥ 99 % purity) and used as follows in the culture media (per litre): 4.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 1.7 g citric acid, 40 mg EDTA, 1.2 g $MgSO_4 \cdot 7H_2O$, and 10 mL of trace elements solution. The hydrolysate was supplemented with (per litre): 10 mL of $MgSO_4 \cdot 7H_2O$ (100 g/L), 1.5 g KH_2PO_4 and 0.6 mL of trace elements solution in order to avoid nutritional deficiencies. The pH was adjusted to 6.8 with KOH (4 M). Concentrated juice of plum waste or solutions containing a blend of sugars simulating its composition were used as feed during the fed-batch phase.

The inocula (10 % v/v) were prepared by inoculating one cryovial into a flask containing 250 mL of the mineral medium supplemented with 20 g/L of glucose and cultured for 12 h at 30 °C and 170 rpm in a rotary shaker (Infors HT, Ecotron) (Izaguirre et al., 2019).

2.3. Fed-batch production of P(3HB) using OFMSW hydrolysate

Fed-batch fermentations were carried out in a 5 L stirred BIOSTAT bioreactor (Sartorius AG, Germany) containing 2.5 L of basal medium or supplemented hydrolysate (or simulated hydrolysate when referred). Temperature was maintained constant at 32 °C using a recirculation cooler (Frigomix 1000; Sartorius AG, Germany). The dissolved oxygen (DO) set-point was 20 % saturation. This value was maintained by cascade control of the stirring speed (initial speed was set at 250 rpm) and also by a constant air flow of 3.6 $L \cdot min^{-1}$. The pH was controlled

automatically at 6.8 with HCl (4 M) and NH_4OH (20 % v/v). The base served as nitrogen source throughout the fermentation, thus polymer accumulation was promoted by phosphate limitation. Foam generation during the fermentation was controlled through manual addition of antifoam (polypropylene glycol; Steinheim, Germany). Data were collected using the software MFCS DA (Sartorius AG, Germany). Samples (10 mL) were taken periodically for analysis of sugar and phosphorous consumption, cell growth and P(3HB) production.

Pulse feeds of glucose (cultivation *A*), simulated plum concentrate (cultivations *B* and *C*) or real waste plum concentrate (cultivations *D* and *E)* were added manually when needed, based on the increase in the DO value.

2.4. Analytical methods

Bacterial growth was controlled by measuring the optical density (OD_{600}) of samples, taken throughout the fermentation, at 600 nm in a spectrophotometer (Shimadzu UV-2401PC). To determine the cell dry weight (CDW), 1.2 mL of culture broth was harvested by centrifugation (Sigma 1-15PK) at 14000 rpm for 5 min in 1.5 mL microtubes, previously dried and weighed. The liquid phase (1.0 mL) was stored at -20 °C for analyzing sugars and phosphate. The impurities of solid phase were eliminated by washing with distilled water (1 mL) and the resulting pellet was dried in an oven (Selecta 200) at 60 °C until constant weight. Dry cells were stored at 4 °C for further analysis of P(3HB) content.

Moisture and dry matter of the OFMSW were determined by heating the sample at 105 °C until constant weight.

The amount of sugars and phosphate in both, fermentation samples and OFMSW hydrolysate, were analyzed using a liquid chromatography system (Agilent Technologies 1260 Infinity II), equipped with an auto sampler (Agilent 1260 Vialsampler G7129A) and coupled to refraction index detector (Agilent 1260 RID G7162A). The separation of the different compounds was performed on a Hi-Plex H (300 mm x 7.7 mm) column. The mobile phase consisted of a 5 mM H_2SO_4 solution.

The operating conditions were: column temperature 65 °C, injection volume 2.4 µL and flow rate 0.5 mL·min^{-1}. All analyses were performed in duplicate and standards were used for identification and quantification of the compounds.

Total quantity of carbon, nitrogen and hydrogen was measured using a Leco CHNS-932 elemental analyzer.

To determine the P(3HB) concentration, 1.2 mL of samples from the culture broth were taken and centrifuged. The cell pellet was subjected to methanolysis as previously described by Izaguirre et al. (2019), and the monomers concentration quantified by Gas Chromatography-Mass Spectrometry (GC-MS) using a 7890 A gas chromatograph (Agilent Technologies, Avondale, PA, USA) coupled to a 5975 C electron impact ionization mass spectrometer and 7683 B Agilent autosampler. The chromatographic separation was carried out in the splitless mode (2 min at 120 °C) in a HP-5MS UI capillary column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies, Avondale, PA, USA). The oven temperature program used for the separation was as follows: the initial temperature was 40 °C, then increased at 10 °C·min^{-1} until 60 °C, after that maintained 4 min and finally increased at 50 °C·min^{-1} until 230 °C. The helium carrier gas flow was maintained constant at 1 mL·min^{-1} and the injection volume was 2 µL. The qMS transfer line, ion source and quadrupole temperatures were 300 °C, 230 °C and 150 °C, respectively. Scan mode with mass range of 40-600 *m/z* was used for the measurements. The 3-methylhydroxybutyrate standard (Sigma Aldrich, Steinheim, Germany) was used for the calibration curves to quantify the polymer extracted.

3. Results and discussion

In recent years, the use of sugar-rich hydrolysates of waste materials in fermentation processes to produce high-value products such as P(3HB) has become popular. This fact originates from the need to reduce both the production cost of these products and the amount of wastes that are disposed in the environment. The hydrolysate used in this work was recently validated by Izaguirre et al. (2019) for P(3HB) production by *B. sacchari* DSM 17165. This strain has the ability to consume

hexoses and pentoses simultaneously at specific rates that depend on the hexose/pentose ratio (Raposo et al., 2017). This is a valuable feature since the main sugars of the OFMSW hydrolysate are glucose (19 $g \cdot L^{-1}$), xylose (3.4 $g \cdot L^{-1}$) and arabinose (0.5 $g \cdot L^{-1}$). The results obtained in the above cited work confirmed *B. sacchari* DSM 17165 as a good candidate for the bioreactor studies.

3.1. Fed-batch cultivations: preliminary study

Some recent studies where *B. sacchari* DSM 17165 was used for the production of P(3HB) have been published (Ashby et al., 2018; Cesário et al., 2014; de Sousa Dias et al., 2017; Rodríguez-Contreras et al., 2015). For example, Cesário et al. (2014), using a wheat straw hydrolysate as carbon source, achieved a biomass concentration of 136 $g \cdot L^{-1}$ as well as a P(3HB) accumulation of 72 % and a volumetric productivity of 1.5 $g \cdot (L \cdot h)^{-1}$ under phosphorous limitation. In most cases, this strain showed better results under phosphorous rather than nitrogen limitation (Silva et al., 2004). In the light of the results obtained by these authors, polymer accumulation under phosphorous limitation was also the strategy followed in the present work.

To assess the behaviour of *B. sacchari* DSM 17165 during cultivations in the fed-batch mode, an assay was conducted (cultivation *A*) starting with the basal medium described in section 2.2. This medium was supplemented with 20 $g \cdot L^{-1}$ of glucose as carbon source. During the cultivation, manual pulses of a concentrated glucose solution (500 $g \cdot L^{-1}$) were added in order to maintain a high level of carbon and achieve high cell density. The time course of the CDW, P(3HB) concentration ($g \cdot L^{-1}$), as well as the glucose and phosphorous concentrations in the medium are shown in Fig. 1.

A maximum CDW of 62 $g \cdot L^{-1}$ was obtained after 47 h of fermentation. Phosphorus was depleted at 26 hours of cultivation time, after which polymer production increased rapidly to reach 38 $g \cdot L^{-1}$. The yield of polymer on sugar consumed ($Y_{P/S}$) and the volumetric productivity obtained over the whole cultivation time ($Prod_{vol}$) were 0.20 ($g_{P(3HB)}/g_{sugar}$) and 0.81 g $P(3HB) \cdot L^{-1} \cdot h^{-1}$) respectively (Table 1). These

values served as reference to assess the performance of subsequent cultivations using real hydrolysate or real plum concentrate.

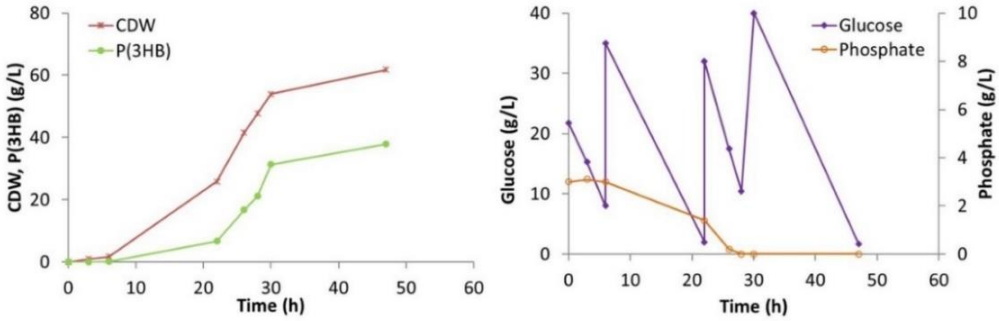


Fig. 1: Time course of CDW and P(3HB), glucose and phosphate concentrations during fed-batch cultivation *A* on mineral medium supplemented with 20 g·L^{-1} of glucose; extra glucose was added manually in pulses.

3.2. Feeding strategies

Due to the limited reactor working volume, operation in the fed-batch mode requires the use of concentrated feed solutions to be able to reach high cell concentrations and also to prolong the production period. If the OFMSW hydrolysate was used as feed, it would be necessary to remove a portion of its water content in order to increase its sugar concentration. This additional step would imply high economic and energy costs. However, some authors have highlighted the idea of using fruit waste juices as carbon source in ethanol, baker's yeast and bacterial cellulose production (Dhillon et al., 2013; Diboune et al., 2019; Kosseva, 2017). In this study, following the same approach, a waste plum concentrate was used as feed during the cultivation. As mentioned in section 2.1, the main sugars in the plum concentrate were glucose and fructose. This is a positive factor, since *B. sacchari* DSM 17165 is able to uptake fructose (Brämer et al., 2001).

Feed addition was performed differently during the day and during the night. During day time, the feed pulses were added manually when a decrease in sugar concentration or an increase in dissolved oxygen (DO) was detected in the culture

medium. At night an external peristaltic pump was programmed to add pulses of 250 mL feed every 3.5 h, so that the culture medium always had an adequate concentration of carbon. The time interval between additions was set according to the sugar consumption rates which had been previously determined. This strategy turned out to be rather effective for P(3HB) production as discussed below.

3.3. Cell growth and polymer production in fed-batch cultivations

In order to obtain high productions of P(3HB), high cell concentrations should be reached during the initial phase of the culture. For that, the growing cells require a balanced medium composition where some nutrients, namely nitrogen, are essential. On this respect, the OFMSW hydrolysate has a rather complete nutrient composition, where in addition to previously mentioned sugars, other elements such as calcium, magnesium and nitrogen stand out (Izaguirre et al., 2019). Precisely, the high nitrogen content (1.5 $g \cdot L^{-1}$) makes the hydrolysate particularly suitable for use as an initial medium in the batch phase, since this element is essential for cell growth. On the other hand, to promote the production of P(3HB) the feed must have a high carbon content and at the same time have a limitation in at least one essential nutrient. As a matter of fact, the plum concentrate meets this criterion very well, since it has a high concentration of sugars and its content in nitrogen (0.9 $g \cdot L^{-1}$) and phosphorus (0.3 $g \cdot L^{-1}$) is low.

At bioreactor scale, prior to combining the use of the OFMSW hydrolysate as an initial medium and the plum concentrate as feed (cultivation *E*), experiments were conducted using mixtures of commercial sugars in which the composition of the hydrolysate (cultivations *B* and *D*) and of the plum concentrate (cultivations *B* and *C*) were simulated. The time course of CDW and P(3HB) produced as well as sugar and phosphate consumption for cultivations B C and D is given on Fig 2. Rate and yield parameters calculated for each cultivation are given on Table 1.

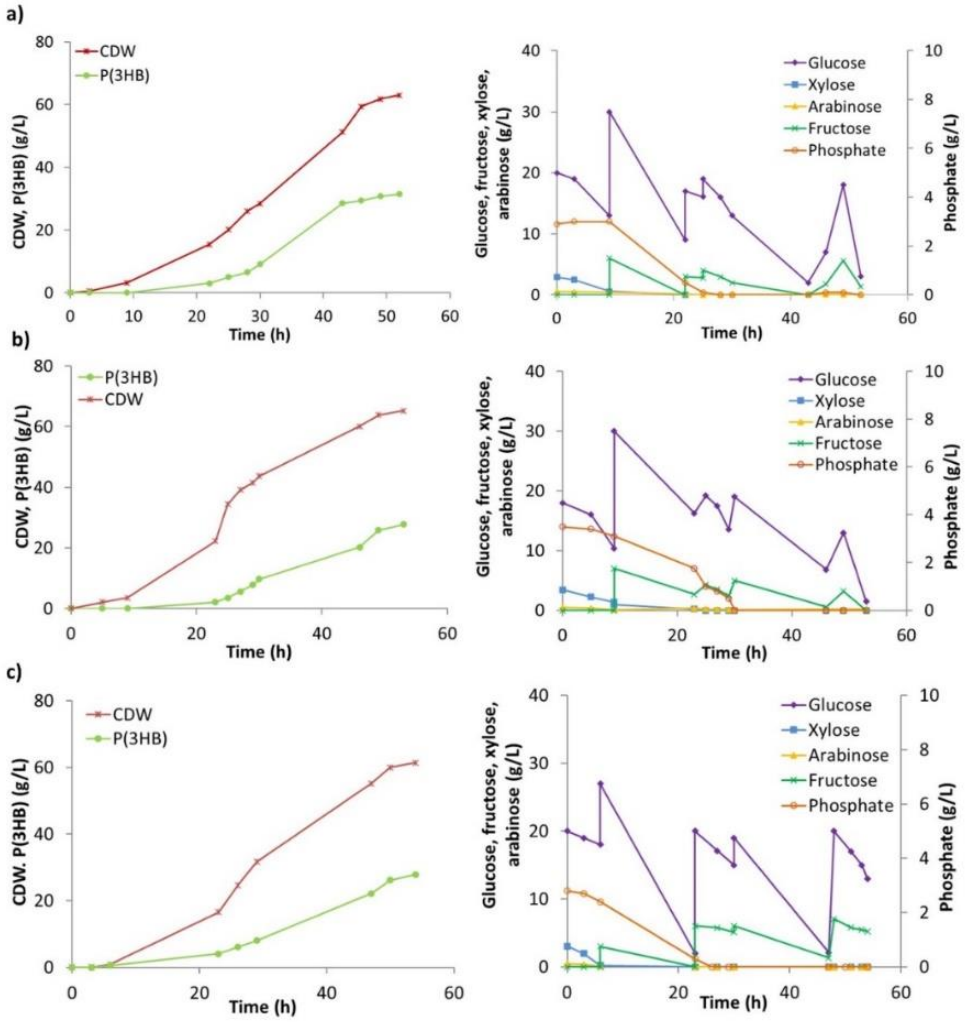


Fig. 2: Time course of CDW, P(3HB), sugars and phosphate concentrations during fed-batch cultivations on a) basal medium supplemented with a simulated hydrolysate with pulses of simulated plum concentrate (cultivation *B*), b) OFMSW hydrolysate with pulses of simulated plum concentrate (cultivation *C*) and c) basal medium supplemented with a simulated hydrolysate with pulses of real plum concentrate (cultivation *D*).

Fig 2 shows that the rate of P(3HB) production when using simulated or real plum concentrate as feed (cultivations *B, C* and *D*) is lower as compared with cultivation *A* using glucose. This is because, even though glucose and fructose are consumed simultaneously , the rate of glucose consumption is higher than that of

fructose, showing thus that *B. sacchari* has a clear preference for glucose has already reported by other authors (Nascimento et al., 2016). These results lead to lower polymer productivities in cultivations *B*, *C* and *D* as given in Table 1.

Table 1: *Burkholderia sacchari* cultivation parameters calculated for each experiment based on the total sugar consumption, corresponding biomass concentration and maximum polymer concentration.

Cultivation	Initial medium	Feed	Sugar consumed (g/L)	CDW_{max} (g/L)	$P(3HB)_{max}$ (g/L)	X_{res} (g/L)	P(3HB) (%)	Time (h)	$Prod_{vol}$ ($g \cdot (L \cdot h)^{-1}$)	$Y_{p/s}$ (g/g)
A	Basal	Glucose	155	62	38.0	23.8	61.0	47	0.81	0.20
B	Simulated hydrolysate	Simulated plum concentrate	160	63	31.5	31.5	50.0	52	0.61	0.20
C	Hydrolysate	Simulated plum concentrate	180	65	27.7	37.3	42.5	53	0.52	0.15
D	Simulated hydrolysate	Plum concentrate	150	61	27.8	33.2	45.4	54	0.51	0.19
E	Hydrolysate	Plum concentrate	195	71	30.0	41.0	43.0	51	0.59	0.15

On the other hand, it is observed that when the hydrolysate is used as initial medium and simulated plum concentrate as feed (cultivation *C*), the attained CDW value is slightly higher (65 g·L^{-1}) than that obtained on the simulated hydrolysate (cultivations *B* and *D*) (Fig. 2). This is clearly perceived already after 20 h cultivation. These observations, combined with the high values of residual biomass (X_{res} = CDW – Conc. P(3HB)) (37.3 g·L^{-1}) and of consumed sugars (180 g·L^{-1}) after 53 h, seem to indicate that the OFMSW hydrolysate promotes cell growth, disfavouring polymer production. The lower yield of polymer on sugar, $Y_{P/S}$ = 0.15 $g_{polymer} \cdot g_{sugar}^{-1}$, calculated for cultivation C is an indication of that. Similar results were obtained by (Al-Battashi et al., 2018) using waste paper with *B. sacchari* DSM 17165. Also, it is interesting to see that with the real plum concentrate and simulated hydrolysate (cultivation *D*), the total amount of sugars consumed, the yield of polymer on sugar and the residual biomass are similar to those using the simulates (cultivation *B*). This fact indicates that the real plum concentrate does not negatively affect both the growth and production of P(3HB) by *B. sacchari.*

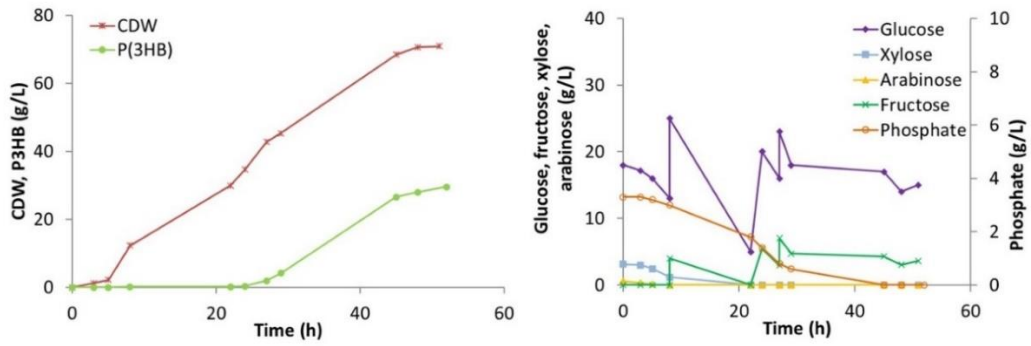


Fig 3: Time course of CDW, P(3HB), phosphate and sugars concentrations during a fed-batch cultivation on real OFMSW hydrolysate with pulses of real waste plum concentrate (cultivation *E)*.

When the hydrolysate was used as initial medium and the real plum concentrate as feed solution (cultivation *E*), a maximum CDW of 71 g·L^{-1} after 51 h, with a P(3HB) accumulation of 30 g·L^{-1} was obtained (Fig. 3). In spite of the increase in CDW and

P(3HB) production, the yield of polymer on sugars consumed ($Y_{p/s}$ (g/g)) was lower (0.15 $g \cdot g^{-1}$, *c.f.* Table 1).

This can be ascribed to both the hydrolysate and plum concentrate favouring biomass production in detriment of P(3HB) accumulation, as indicated by the high value of residual biomass (X_{res} = 41 $g \cdot L^{-1}$). The P(3HB) accumulation in cultivation *E* (43 %) was comparable to the values achieved with the combination of i) real hydrolysate and simulated plum concentrate and ii) simulated hydrolysate and real plum concentrate. However, it was lower than with the combination of simulated hydrolysate and simulated plum concentrate. It is worthy to note that the P(3HB) volumetric productivity in cultivation *E* (0.59 $g \cdot (L \cdot h)^{-1}$) attained very similar values to the ones obtained with the simulated initial medium and simulated feed (cultivation *B).*

This propensity to form biomass instead of polymer with the available sugars is most probably due to the N and P content, in particular in the OFMSW hydrolysate. To promote earlier polymer formation in the assays using OFMSW hydrolysate as substrate, the phosphorous content should be reduced in the supplemented mineral medium. Another reason for the preferential biomass formation when using real hydrolysates is the presence of peptides and amino acids derived from the hydrolysis of proteins in the OFMSW. For some microorganisms, these biomolecules are easier assimilated compared to an inorganic nitrogen source such as NH_4^+. This could also explain why cell growth is faster with hydrolysates than with simulated media. A similar effect was reported by Al-Battashi et al. (2018), when assessing different sources of nitrogen in the production of P (3HB). They observed that the use of organic sources promoted the growth of the microorganism rather than the production of the polymer.

As pointed earlier, there are several reports on the use of *B. sacchari* strains to produce P(3HB) using hydrolysates as carbon source (Cesário et al., 2014; Silva et al., 2004; Pradella et al., 2010). The productivities and yields were generally higher than those reached in the present work. This could be due to the fact that they used

lignocellulosic biomass of only a single type, while in this study a more complex and heterogeneous feedstock (OFMSW) was used. In contrast, the literature available on the use of complex substrates for P(3HB) production is limited. For example, Cerrone et al. (2015) reported a P(3HB) production of 44.5 $g \cdot L^{-1}$ with a polymer content of 33 % using mannitol rich ensiled grass press juice waste.

The results obtained in the present work suggest that the combination of the OFMSW hydrolysate as initial medium with the waste plum concentrate as feed solution is an attractive approach to produce P(3HB) from C-rich waste streams. However, it is still necessary to search for new ways to make the process more efficient towards P3HB production. The straight solution would be to adjust the phosphorous concentration supplemented to the hydrolysate so as to promote an earlier start of polymer production. Another possibility could be to extract the protein fraction of the residues prior to the saccharification of the carbohydrate fraction, applying thus the cascade biorefinery concept. This protein fraction could be upgraded separately to be included as additive in animal feed.

4. Conclusions

In this work P(3HB) was produced from an OFMSW hydrolysate and a residual plum juice stream using *B. sacchari* DSM 17165 at laboratory scale. Due to its balanced nutrient composition the OFMSW hydrolysate was used as initial cultivation medium to attain a high cell density and, subsequently, an off-stream of the production of plum concentrate, rich in sugars, was used as feed solution. The combination of these two feedstocks was shown to be an excellent strategy to reach a high P(3HB) productivity (0.59 $g \cdot (L \cdot h)^{-1}$). Further, it can be claimed that the combined utilization of the OFMSW hydrolysate and of the residual plum juice stream in P(3HB) production will not only decrease the amount of urban waste, but also reduce the environmental impact that would be originated if non-renewable raw materials were used. These results may contribute to create sustainable solutions to manage the municipal waste and inspire the design of production plants based in bio-waste streams, hence contributing to a circular economy.

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MANUSCRIPT 3: Techno-economic and environmental assessment of poly(3-hydroxybutyrate) production from organic fraction of municipal solid waste

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Abstract

The large volume of municipal solid waste produced in cities worldwide is considered to be a major problem. However, its organic fraction may be seen as a promising source of nutrients, especially of carbon, for poly(3-hydroxybutyrate) production. In this study, a techno-economic and environmental process assessment of poly(3-hydroxybutyrate) production from the organic fraction of municipal solid waste was performed, based on previously published laboratory-scale results. To facilitate the analysis, SuperPro Designer® software was used, by means of which the build of a poly(3-hydroxybutyrate) production plant was simulated. The waste processing capacity of the plant was 1 ton·day^{-1} and two scenarios with different fermentation starting mediums were proposed: Scenario (1) organic fraction municipal solid waste hydrolysate, scenario (2) basal medium supplemented with glucose. It was found that both scenarios were economically unfeasible at this scale. The low fermentation yield due to the complexity of the biomass, enzyme cost, labor cost and energy consumption were the factors that most contributed to the infeasibility of scenario 1. However, for scenario 2 these factors were the extraction solvent and also the fermentation yield. Increasing fermentation performance and considering using process waste streams as raw materials to generate other high-value compounds could be the key to increasing process viability. In both scenarios, photochemical oxidation potentially caused by the use of anisole as an extraction solvent and acid rain, potentially caused by the burning of fuels for power generation, were the greatest environmental impacts generated by the plant.

Keywords

Economic and environmental evaluation; *Burkholderia sacchari*; Poly(3-hydroxybutyrate); Circular Economy; Municipal solid waste.

1. Introduction

Due to the development of science and technology, industrial and commercial activities have undergone great growth and alongside these activities the generation

of waste. This can no longer be assimilated by natural cycles and, as a result, significant environmental problems arise. An example of this would be the generation of municipal solid waste (MSW), which has grown massively in recent decades and has become a real worldwide concern (Eurostat, 2018). MSW typically includes the non-hazardous mixture of wastes generated by households, industries, institutions, agriculture and sewage. World production is approximately 2000 million tons per year, where between 34-53 % represents the organic fraction (OF), and is mainly made up of food scraps, paper, garden and forest trimmings (Abad et al., 2019; Braguglia et al., 2018; Qin et al., 2017). Generally, landfill, incineration, composting Mechanical Biological Treatment (MBT) and biogas production are the most widely implemented strategies for organic waste disposal. However, despite technological improvements in waste management, the organic fraction of municipal solid waste (OFMSW) generation per capita continues to increase and its decomposition may generate problems such as methane emissions, leachates, ecosystem alterations and animal diseases among others (Demichelis et al., 2017). In addition, these strategies designed to reduce the environmental problems are relatively expensive (Gaeta-Bernardi and Parente, 2016).

From the perspective of the circular economy, raw materials derived from agriculture have been used for many years. However, other types of waste, including OFMSW also have the potential to serve as feedstock in biorefineries for chemicals, fuels and other materials due to their nutrient rich composition, abundance and low cost (Battista et al., 2020). For example, Barampouti et al. (2019) reviewed the production of bio-ethanol, bio-diesel and biogas from OFMSW. Also, Ghanavati et al. (2015) and Izaguirre et al. (2019) studied the use of OFMSW to produce lipids and Poly(3-hydroxybutyrate) (P(3HB)) respectively. Compared to other organic waste, OFMSW is produced daily in all cities, which gives it an advantage over other waste, since its availability is high throughout the year. Another additional advantage is that it is generated in all urban areas and therefore its transportation to processing sites, such as a P(3HB) production plant, would have a lower environmental impact (Shahzad et al., 2013).

Defining appropriate strategies for the use OFMSW in biotechnological processes is essential and can significantly influence the costs of the process. For example, the use of mild conditions, such as enzymatic hydrolysis, to release the nutrients contained in the biomass prevents the generation of inhibitors and increases productivity. However, the use of enzymes is highly expensive (Amit et al., 2018). This creates a conflict between whether it is cheaper to use enzymes and produce a larger quantity of product, or use strong conditions and save on enzyme purchase. Futhermore, assigning OFMSW to the production of biodegradable plastics would not only reduce the amount of municipal solid waste and plastic debris, but also help to alleviate excessive dependence on petroleum.

One of the major expenses in a fermentative process is the substrate, namely, the carbon and nitrogen source. Replacing these raw materials with organic waste results in big cost savings and at the same time improves the feasibility of the process (de Paula et al., 2017; Obruca et al., 2015). The feasibility of a process depends on many factors, such as the substrate and material costs, labor costs, plant location and taxes among others. In this line, techno-economic studies serve to examine the feasibility of a process. These studies are based on the results obtained previously at lab-scale or at pilot scale experiments by using a process simulation software. The commonly used softwares are SuperPro Designer® and Aspen Plus®.

In this work, the techno-economic feasibility of fermentative production of P(3HB) from the OFMSW by *Burkholderia sacchari* DSM 17165, a strain able to consume glucose, xylose and arabinose simultaneously, was evaluated. For the economic analysis, the whole process was considered including obtaining the substrate, fermentation and extraction of the polymer. The evaluation was based on the lab-scale data obtained from a previous study and was performed using SuperPro Designer® software (Izaguirre et al., 2019).

2. Materials and methods

2.1. Simulation

In this study a process for P(3HB) production from OFMSW was economically and environmentally evaluated. Mass and energy balance requirements for the process were estimated by carrying out a simulation with the aid of the commercial software SuperPro Designer 10®. The P(3HB) production plant was to be built in The Basque Country (Spain) and its lifetime would be 20 years. The construction and start-up phase would be 1 year. According to the Ministerio para la transición ecológica of Spain, each year 877 thousand tons of OFMSW are generated, of which 2 % (175.4 thousand tons) are produced in The Basque Country. This type of waste is selectively collected and 70 % is derived to produce compost and biogas, while the remaining 30 % is disposed of in landfills or incinerated (MITECO, 2017). As reference the processing capacity of the plant was to be 1 ton·day^{-1} and it would operate for 330 days·year^{-1}. The rest of the time up to 365 days the plant was to stop for maintenance and cleaning work.

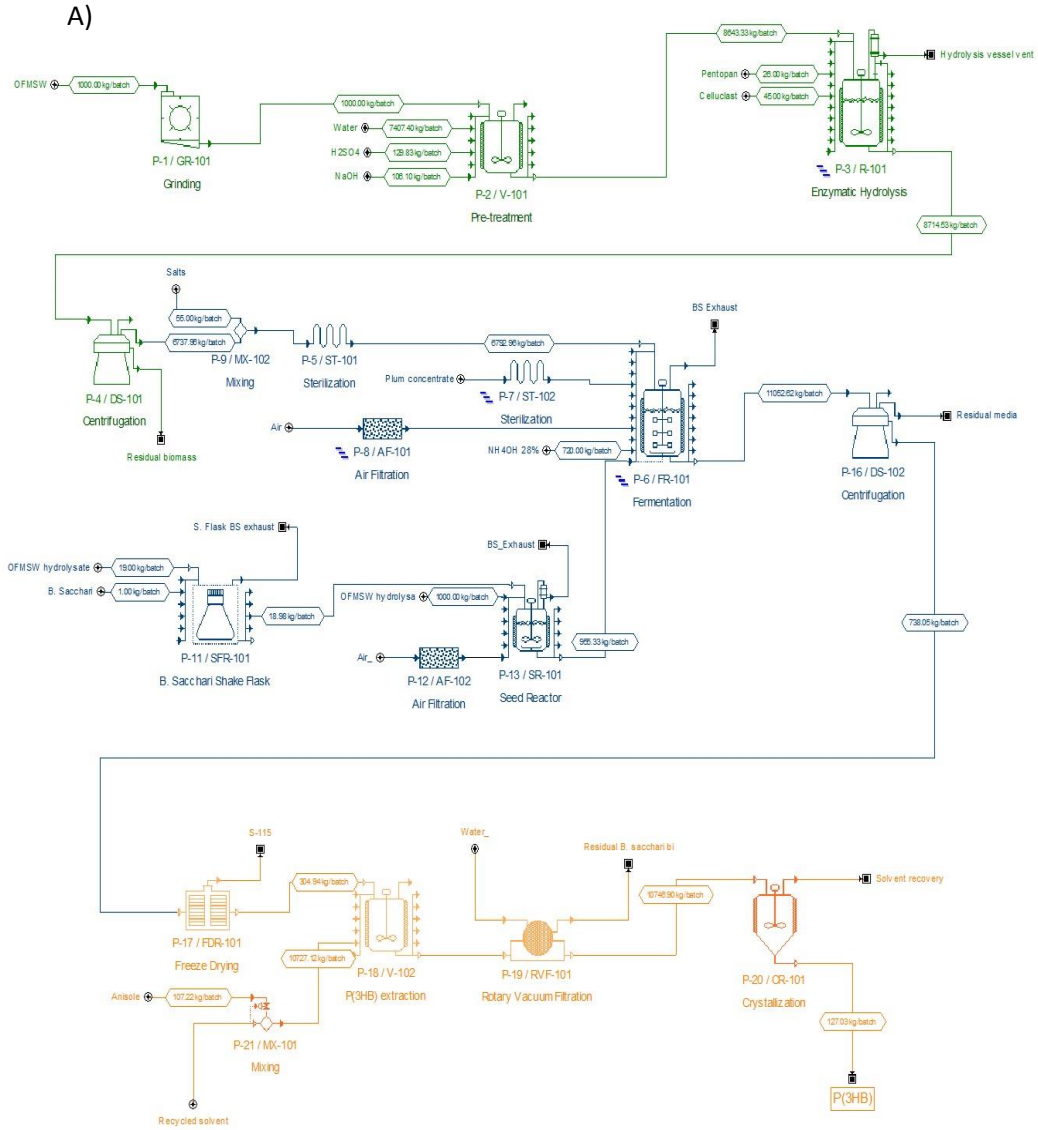
2.2. Scenarios

The industrial plant simulated in this study, was based on the production process of P(3HB) from renewable feedstocks (OFMSW and plum waste juice), developed and reported by the same authors in a previous work (Izaguirre et al., 2019). Two scenarios were analyzed to evaluate the feasibility of the process: Scenario 1 involves the full process. Briefly, in a first step, sugars present in the OFMSW were released by the combination of thermo-chemical pretreatment and enzymatic hydrolysis. In a second step, the hydrolysate obtained was used as initial medium in the fermentative production of P(3HB). During the fermentation, following a fed-batch strategy, the sugar-rich plum waste juice was used as a feed solution to achieve high productivity. Finally, the P(3HB) produced was extracted (figure 1A). In scenario 2, pretreatment and enzymatic hydrolysis steps were eliminated, which is to say the hydrolysate was not used as initial medium to start the fermentation (figure 1B). Instead, the fermentation step was started with a basal medium, whose

composition consisted in salts and glucose and was described in a previous study (Izaguirre et al., 2019). In both cases, plum concentrate was used as feed solution. Comparison of both scenarios served to know to what extent the use of OFMSW as an initial source of nutrients contributed to the final cost of the overall process.

To simplify the flowsheet pieces such as valves and piping were omitted, but were taken into account in the economic analysis.

A)



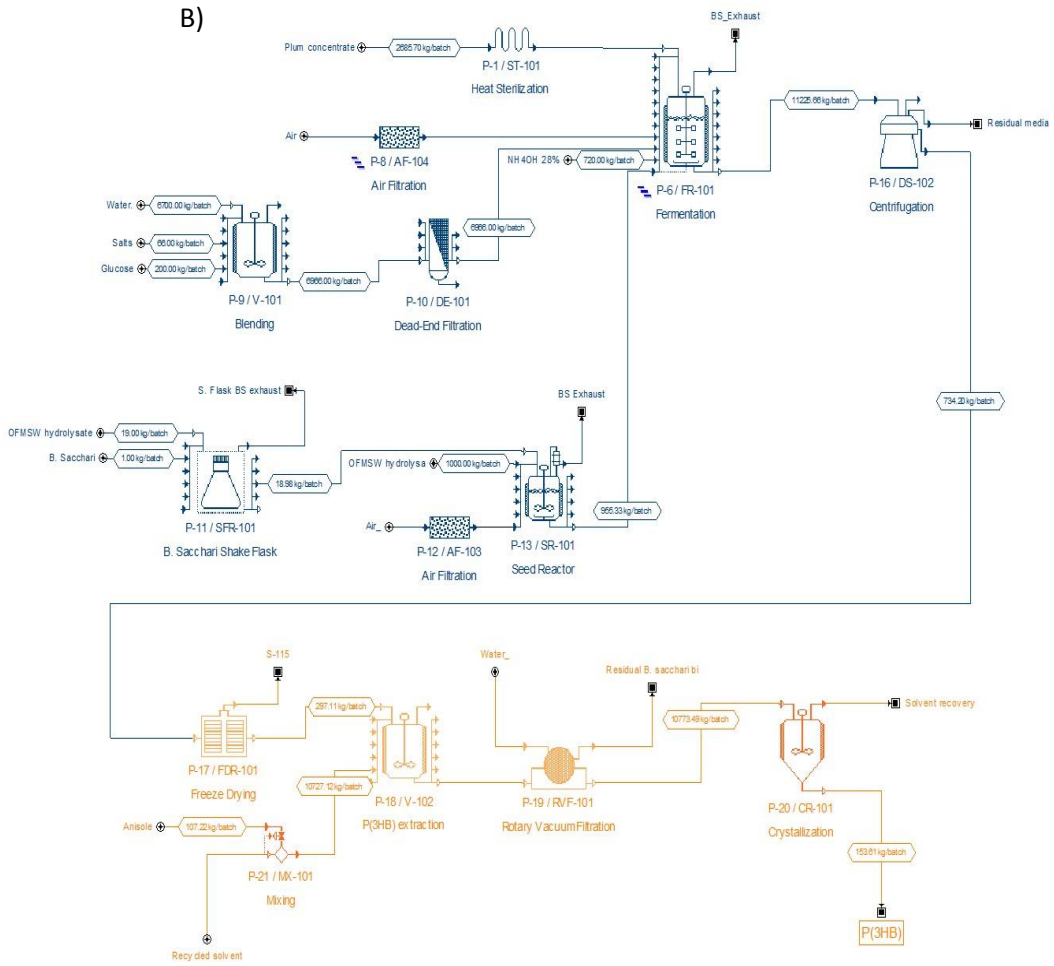


Fig. 1: Flowsheet of the P(3HB) production process. Green: thermo-chemical pre-treatment and enzymatic hydrolysis steps. Blue: Fermentation step. Orange: extraction step. A) Scenario 1 and B) scenario 2.

2.3. Process description

As shown in Fig. 1A, the overall P(3HB) production process includes three main steps: i) OFMSW pre-treatment and hydrolysis, ii) fermentation and iii) separation.

2.3.1. Thermo-chemical pre-treatment and enzymatic hydrolysis

OFMSW was initially ground into smaller size pieces and subsequently mixed with 1 % H_2SO_4 solution at a solid to liquid proportion of 13.5 %. Then, the biomass was pre-treated at a temperature of 121 °C during 60 min in a blending tank (V-101). During pre-treatment, the lignocellulosic structure of the biomass was broken up and made more accessible to the enzymes. The resulting mixture was cooled and neutralized with NaOH in the same tank. After neutralization, the mixture was enzymatically hydrolyzed in a reactor (R-101). The reaction was carried out at 50 °C for 24 h using a blend of 90 $mg \cdot g^{-1}$ substrate and 150 $mg \cdot g^{-1}$substrate of Pentopan 500 BG and Celluclast BG respectively. Finally, the non-hydrolyzed solids were separated by centrifugation, in a disk tank centrifuge (DS-101), and the supernatant was used in the next step. This sub-process was only taken into account in the first scenario.

2.3.2. P(3HB) production: fermentation

The hydrolysate obtained was heat-sterilized and transferred to a fermenter (FR-101) for P(3HB) production and inoculated with a *Burkholderia sacchari* culture (10 % v/v) previously incubated for 12 h, first in a shake flask (SFR-101) and then in a seed reactor (SR-101). The fermentative process was carried out aerobically at a pH 6.8 and 32 °C for 50 h. In the next stage, the solid fraction was separated from the liquid by centrifugation in a disk-tank centrifuge (DS-102) and the P(3HB) rich bacterial biomass was lyophilized in a freeze dryer (FDR-101).

2.3.3. P(3HB) Separation: extraction

The extraction sub-process proposed in this work was previously reported by (Rosengart et al., 2015). The dried biomass was transferred to a blending tank (V-102), which contained anisole at 120 °C. The blend was mixed in the tank for 30 min. The extraction solvent acts by increasing cell permeability and dissolving the released P(3HB). After extraction, the remaining cell biomass was separated by filtration in a rotary vacuum filter (RVF-101). Finally, separation of the polymer from

the solvent was carried out by crystallization in a continuous crystallizer (CR-101). It was assumed that the evaporated solvent was collected to be used again in subsequent extractions.

2.4. Economic analysis

The economic evaluation of both scenarios was performed using SuperPro Designer 10® software, through which the total capital cost, annual production cost and revenue generation can be estimated.

2.4.1. Total capital cost

The total capital cost of the plant involves three components: direct fixed capital (DFC), working capital and start-up validation cost. DFC includes the equipment purchase cost and other direct and indirect costs, such as piping, insulation and engineering among others, related to the construction of the plant. The contribution of each component can be estimated based on the total equipment purchase cost using several multipliers (Petrides, 2015). The price of the equipment is gathered from reputable websites such as Alibaba. The Basque Government favors the creation of new industries; thus, the cost of the land was not taken into account in the economic evaluation. The contribution of the working capital and start-up validation cost to the total capital cost was 1.5 % and 5 % of the DFC respectively.

2.4.2. Annual production cost

Among those factors that contribute most to the annual production cost there are raw materials, namely enzymes, solvents, salts, among others. They also include maintenance and repair, labor, utilities, quality control laboratory, consumables, and waste disposal. The cost of raw materials and consumables was provided by the main suppliers of laboratory equipment. OFMSW collection and transportation was sponsored by the Gipuzkoa Waste Consortium. It was assumed that the plum waste juice was provided by CATAR-CRITT Agroressources (France) and thus the cost of this raw material was not included. Facility dependent costs correspond to

depreciation of the fixed capital investment, equipment maintenance costs, insurance, local (property) taxes and other general expenses. P(3HB) production plant was designed to operate for 20 years and the straight-line method was used to calculate capital depreciation. Equipment maintenance and repair cost was assumed to be 1 % of DFC. In accordance with the local legislation, insurance and taxes were assumed to be 0.04 % and 1.38 % of DFC, respectively. Labor costs are basically the salaries of operators and engineers, to which must be added taxes. For the correct operation of the plant, in scenario 1, six operators and two engineers were necessary, while in scenario 2 the need was for four operators and two engineers. The salary of an operator and engineer was approximately US$ 26,000 and US$ 41,000·year^{-1} respectively. The consumption of each type of utility was estimated based on the mass and energy balance calculated by the simulation software, and their unit costs were obtained from the supplier companies. The cost of the laboratory for quality control was assumed to be 15 % of the total labor cost. Regarding waste disposal, the cost of disposing of organic waste is 0.013 U$·Kg^{-1} and the cost of gaseous emissions is 0.002 $·Kg^{-1}.

2.4.3. Revenue and annual profitability

In both scenarios the main revenue comes from the sale of P(3HB). However, in scenario 1, the undigested OFMSW from the enzymatic hydrolysis was sold as biofertilizer at a price of 0.01 US$·Kg^{-1} and also a waste treatment (0.077 US$·Kg^{-1}) fee was received from the Basque Government. At present, the market price of P(3HB) is 4000 US$·MT^{-1} (Ramos et al., 2019; Stavroula et al., 2020).

The profitability of the process was evaluated based on various indicators, which were calculated by the software during the simulation. These indicators included gross and net profit, gross margin, return on investment (ROI), net present value (NPV) and payback time. Gross profit is the revenue from which the annual operating cost has been subtracted, while net profit also considers depreciation and income tax (20 % in The Basque Country). The return of investment (ROI) evaluates the viability of the investment in line with Eq. (1).

$$ROI\ (\%) = \frac{Net\ profit}{Total\ investment}\ x\ 100 \qquad (1)$$

NPV measures profitability of the process in absolute net terms, which allows one to know if the investment will bring profits. A positive NPV value suggests that the planned investment will make a profit and thus be viable. NPV can be calculated according Eq. (2) (Van Dael et al., 2015).

$$NPV = \sum_{n-1}^{T} \frac{CF_n}{(1+i)^n} - I_0) \qquad (2)$$

Where T is the lifetime of the investment, CF_n the difference between revenues and costs in year n, I_0 the initial investment and i the discount rate. The payback time refers to the time required to recover the capital investment and is calculated by using the following Eq. (3).

$$Playback\ time\ (years) = \frac{total\ investment}{net\ profit\ per\ year} \qquad (3)$$

To determine the cash flow patterns during the plant´s lifetime, cumulative cash flow was calculated. Various discount rates were also investigated in order to show their effect on profitability. The patterns were plotted using Microsoft Excel 2010.

2.5. Environmental analysis

To estimate the environmental impact of the process, an algorithm (waste reduction (WAR)) developed by the U.S. Environmental Protection Agency (EPA) was used. The algorithm is based on the calculation of the potential environmental impact (PEI) and it is divided into eight impact categories: human toxicity potential by ingestion (HTPI), human toxicity potential by dermal exposure and inhalation (HTPE), aquatic toxicity potential (ATP), acidification or acid-rain potential (AP), terrestrial toxicity potential (TTP), photochemical oxidation potential (PCOP), global warming potential (GWP) and ozone depletion potential (ODP).

3. Results and discussion

3.1. Techno-economic analysis

3.1.1. Total capital cost

The capital cost comes out as US$ 7,418,949 and US$ 5,549,004 for scenarios 1 and 2 respectively. As mentioned in section 2.4.1., this cost encompasses DFC, working capital cost and start-up validation cost. DFC included the purchase of equipment and its installation, engineering cost, the plant building and other related costs. The size and number of the components and equipment were estimated based on the mass and energy balance of the simulated process.

Table 1 details the bare minimum equipment used in both scenarios as well as their characteristics and cost. The equipment cost was US$ 1,066,000 for scenario 1 and US$ 764,000 for scenario 2. This difference is due to the fact that the second scenario did not contemplate the use of OFMS as the initial media and therefore the pretreatment and enzymatic hydrolysis steps were not performed. In both scenarios the equipment that contributed most to the cost (US$ 100,000) were the fermentors (FR-101). This is in line with findings by Kwan et al. (2015), Leong et al. (2017) and Mudliar et al. (2008), who also observed a greater contribution of this type of item in the total equipment costs. Table 2 showed the other costs related with the building of the P(3HB) production plant. The contribution of these costs to DFC were estimated based on the product between the equipment purchase cost or direct/indirect costs with several multipliers, which were obtained from empirical data. The working capital represents the expenses derived from the initialization of the plant and the operational training and was calculated at 1.5 % of the DFC. Start-up cost was assumed to be US$ 106,293 and US$ 61,843 for scenario 1 and 2 respectively.

Table 1: Description and purchase cost of the main equipment for scenario 1 and scenario 2.

Equipment code	Equipment name	Units	Size/Capacity	Cost (US$)	
				Scenario 1	Scenario 2
GR-101	Grinder	1	3,000 kg/h	26,000	0
V-101	Blending tank	1	10,000 L	26,000	26,000
R-101	Stirred reactor	3	10,000 L	135,000	0
FR-101	Fermentor	3	15,000 L	300,000	300,000
ST-101	Heat sterilizer	1	1,681 L/h	20,000	20,000
ST-102	Heat sterilizer	3	52 L/h	60,000	0
SFR-101	Shake flask rack	1	24 L	10,000	10,000
SR-101	Seed reactor	1	1,500 L	35,000	35,000
FDR-101	Freeze dryer	1	433 kg	41,000	41,000
V-102	Blending tank	1	15,000 L	54,000	54,000
RVF-101	Rotary vacuum filter	1	15 m^2	52,000	52,000
CR-101	Crystalizer	1	5,000 L	30,000	30,000
AF-101	Air filter	1	32,000 L/h	1,000	1,000
AF-102	Air filter	3	460,000 L/h	3,000	3,000
DS-101	Disk-stack centrifuge	1	2,174 L/h	30,000	0
DS-102	Disk-stack centrifuge	1	1,839 L/h	30,000	30,000
DE-101	Dead-end filter	1	46 m^2	0	10,000
	Unlisted equipment			213,000	152,000
TOTAL				1,066,000	764,000

Table 2: Direct fixed capital (DFC) of the P(3HB) production plant.

Type	Component	% of DFC*	Cost (US$)	
			Scenario 1	**Scenario 2**
Direct	Equipment purchase	15	1067000	759000
	Installation	3	230000	245000
	Process piping	10	709000	489000
	Instrumentation	6	424000	324000
	Insulation	1	67000	44000
	Electrical facilities	2	137000	101000
	Buildings	6	388000	307000
	Yard improvement	2	137000	103000
	Auxiliary facilities	9	644000	479000
Indirect	Engineering	14	951000	713000
	Construction	19	1331000	998000
Other	Contractor´s fee	5	316000	228000
	Contingency	8	565000	368000
		Total	6966000	5158000

* The value of percentages of each component was based on the total equipment purchase cost (PC) using several multipliers (Petrides, 2015).

3.1.1. Production cost

Table 3 shown the annual production and maintenance costs of both scenarios. When they were compared, it was observed that the operating cost of scenario 1 was 28 % higher than that of the second one. This difference can be attributed to the pre-treatment and enzymatic hydrolysis steps, which were only present in the first scenario. Among those factors that contributed most to increasing the production cost in scenario 1 were the price of enzymes, the heating/cooling utilities (pre-

treatment) and the labour cost, since there were two workers more than in scenario 2. In both scenarios, the main operating cost was the facility-dependent cost (approximately 50 %), which accounted for costs related to the use of a facility, namely equipment maintenance, depreciation, and miscellaneous costs (insurance, local taxes and factory expenses). Utilities such as heat transfer agents and power used in either process operation also contributed significantly to the operating cost, particularly in the first scenario (20 %), since pre-treatment was performed at high temperature and involved additional steps. The cost of carbon source is typically one of the major cost contributor in fermentative processes (Esteban and Ladero, 2018; Rodriguez-Perez et al., 2018). However, in this study, the main carbon source (plum concentrate) cost was not taken into account in the analysis, since this was kindly provided by a bioresidues upgrader company (CATAR-CRIT Agroressources, France). It was obtained by extrusion from fruit waste; thus, it is an inexpensive and easily obtainable carbon source. Waste produced was estimated by the program as part of the simulation. The cost of gas emissions during fermentation was 0.002 US$·Kg^{-1}, whereas the cost of treatment of aqueous waste from fermentation was taken to be 0.013 US$·Kg^{-1}.

Table 3: Annual production cost of P(3HB) production plant for scenario 1 and scenario 2.

	Scenario 1			Scenario 2		
Item	**Annual quantity**	**Cost (USD/Unit)**	**Cost (USD/year)**	**Annual quantity (MT^{-1})**	**Cost (USD/Unit)**	**Cost (USD/year)**
Raw materials						
Ammonia	264.5 MT	20	5,290	264.5 MT	20	5,290
Anisole	48.8 MT	2120	103,456	59.1 MT	2120	125,294
Celluclast	16.1 MT	6270	100,947			
Glucose	65.6 MT	400	26,240	137.0 MT	400	54,800
Sodium hydroxide (0.5 M)	9,539.3 MT	10	95,393	9,000.1 MT	10	90,001
Pentopan BG	9.3 MT	9200	85,560			
Salts	19.6 MT	800	13,280	23.6 MT	800	18,880
Sodium hydroxide	37.9 MT	300	11,370			
Sulfuric acid	46.3 MT	70	3,241			
Total			444,777			294,265
Consumables						
2000 mL shake flask	214 item	1.8	385.2	214 item	1.8	385.2

Dead end filtration membrane				8 m^2	400	3319
Total			385.2			3704.2
Laboratory quality control						
Total			19,605.0			26,995.0
Utilities						
Electricity	1,159,122 $kW \cdot h^{-1}$	0.11	127,503.4	789,424.0 $kW \cdot h^{-1}$	0.11	86,836.6
Steam	8738.0 MT	12	104,856.0	4,885.0 MT	12	58620.0
Cooling water	121,580.0 MT	0.05	6,079.0	75,747.0 MT	0.05	3787.4
Glycol	509,864.0 MT	0.35	178,452.4	132,002.0 MT	0.35	46,200.7
Total			411,490.8			195,444.7
Facility dependent						
Total			969,449			761,943.0
Labour dependent						
Total			245,065			194,598.0

Waste treatment/disposal						
Aqueous liquid	3,682.3 MT	13	47,869.9	3,745.4 MT	13	48,690.2
Gas emissions	9,749.6 MT	2	19,499.2	10,051,7 MT	2	20,102.0
Total			67,369.1			68,792.2
Total (operating cost)			2,158,141.1			1,545,742.1

3.1.1. Revenue and annual profitability

Table 4: total annual revenue generated in scenario 1 and scenario 2.

Item	**Scenario 1**			**Scenario 2**		
	Annual quantity (MT)	**Price (US$/Unit)**	**Revenue (US$/year)**	**Annual quantity (MT)**	**Price (US$/Unit)**	**Revenue (US$/year)**
P(3HB)	45	4000	180,000	55	4000	220,000
Biofertilizer	706	10	7,060			
Waste treatment	357	77	27,489			
Total			214,549			220,000

As shown in Table 4, the total revenues obtained throughout the year in scenario 1 and 2 were US$ 214,549 and US$ 220,000, respectively. In both scenarios, revenue came from sales of P(3HB) and from the service provided by the OFMSW treatment. It should be noted that in scenario 1 there was also an extra income from the sale of the residual biomass flow from enzymatic hydrolysis as biofertilizer. Obtaining a higher amount of P(3HB), in the second scenario, was mainly due to the higher yield achieved by the use of a simple salt medium supplemented with glucose to start fermentation. The unit price of P(3HB) and biofertilizer was 4 US$·Kg^{-1} and 0.01 US$·Kg^{-1} respectively, while the fee received from The Basque Government for the treatment of OFMSW was 0.077 US$·Kg^{-1}.

Table 5: Process profitability indicators of scenario 1 and scenario 2.

Indicator	**Scenario 1**	**Scenario 2**
Unit production cost (US$/year)	48	25
Gross profit (US$/year)	-1,943,000	-1,289,000
Net profit (US$/year)	-1,282,000	-793,000
Gross margin (%)	-890	-588
ROI (%)	-17	-14
NPV at 7 % (US$)	-18,213,000	-12,163,000
Playback time (years)	N/A	N/A

The profitability of scenario 1 and scenario 2 was evaluated using several indicators whose value is reflected in Table 5. It was found that both scenarios were economically infeasible, since the NPV and ROI had negative values. Another factor that indicated that the process is not viable was the production cost of P(3HB), which in both scenarios was much higher than its actual market price. These prices are not competitive with those obtained by other authors in similar processes (Al-Battashi et al., 2019; Dietrich et al., 2017; Vandi et al., 2018).

Fig. 2 shows the behavior of NPV (7 % of discount rate) during the project lifetime. For both scenarios, NPV remains negative, which indicated an unfavorable economic outlook in which the expenses are higher than the income. One factor that

influenced the non-viability of the process was the use of OFMSW hydrolysate as a starting medium in fermentation. Hydrolysate production involved some steps such as dilute acid pretreatment and enzymatic hydrolysis where expensive raw materials (enzymes) and high energy were used. This influence is clearly seen when comparing the operating cost of both scenarios, since in scenario 2 OFMSW hydrolysate was not used as the initial media. Another factor that weighed heavily on the unfeasibility of the process, and occurred in both scenarios, was the low fermentation yield. This was primarily because both OFMSW and Plum concentrate were complex and heterogeneous substrates consisting of a mixture of residues or compounds. In fact, other research has also reported that the use of complex substrates can adversely affect fermentation yield (Ghanavati et al., 2015; López-Gómez et al., 2019).

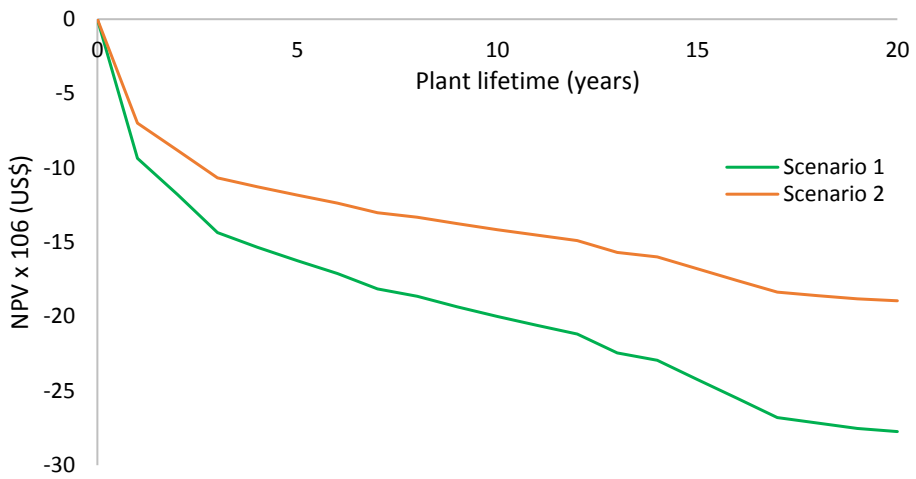


Fig. 2: Net Present Value of the P(3HB) production process over the project lifetime for both scenarios.

The separation and purification step also supposed a considerable cost, especially due to the large amounts of solvent (anisole) that were required. However, in this simulation the recovery of the solvent after the extraction of P(3HB) was considered for reuse in subsequent cycles. Solvent losses through evaporation were also taken into account for a more realistic scenario.

The results indicated that the process was not economically feasible, although some alternatives to increase profits could be proposed. For example, the waste and emissions generated during the process could be used for the production of other valuable products such as biogas, CO_2, H_2 and proteins. Increasing the scale of the plant could also improve profitability as indicated by (Choi, 1999).

Furthermore, instead of using the sugars present in OFMSW to produce P(3HB), they could be used to produce hydroxymethylfurfural (HMF), levulinic acid and other compounds which is highly regarded as a building block in the manufacture of other compounds of interest (Nzediegwu and Josée, 2020; Tulaphol et al., 2020)

In the present techno-economic evaluation, as well as in other similar evaluations, only production costs have been taken into account at a micro level, that is at the production plant level. Nevertheless, it has not been taken into account that using waste as raw material could be a good way of saving money because not using it generates additional costs. Nor has it been borne in mind that the material produced is biodegradable and has less impact after its useful life and the cost of its safe removal will be less. From a macro point of view and taking all these factors into account, it is possible that this process makes more sense, depending on one's perspective.

3.2. Environmental analysis

As described above, the potential environmental impact (PEI) of the P(3HB) production process was calculated using the WAR tool. The environmental impact results of the 8 categories were presented as PEI leaving the system per kg of P(3HB) stream (Fig. 3). According to the results obtained, photochemical oxidation potential (PCOP), due to the use of anisole solvent, generated the highest impact. The burning of fuels for power generation for the plant's operation also had a significant impact, as shown by indicators of acid rain potential (AP) and global warming (GWP). Nevertheless, the impact of energy production could be reduced if the use of an energy mix with a higher percentage of renewable energy is promoted. During the process there were no toxic products used or generated, therefore the potential for

toxicity in humans and in aquatic and terrestrial ecosystems was very low. Halogenated hydrocarbons were not used either, so the potential impact of ozone depletion was zero.

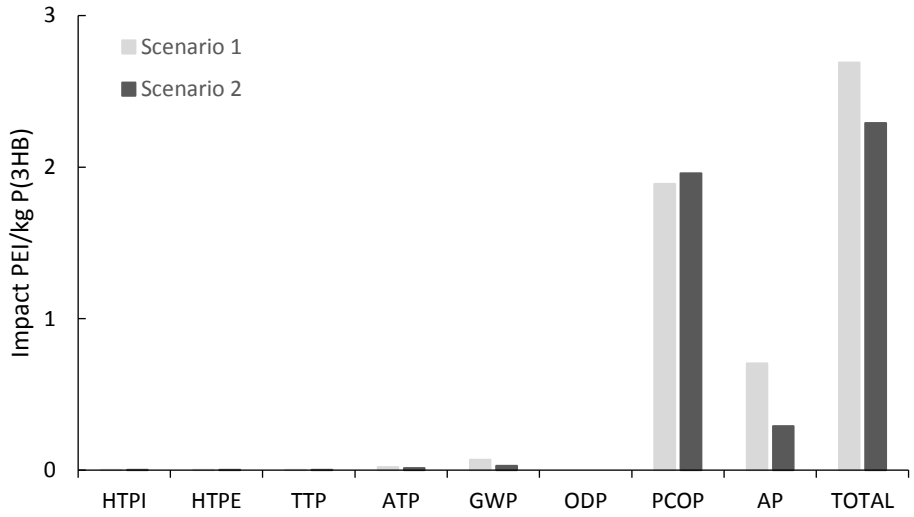


Fig. 3: Potential environmental impact (PEI) per Kg of P(3HB) produced.

Comparing both scenarios, the GWP and AP categories were higher in scenario 1 due to the higher energy consumption in the pretreatment and hydrolysis stages, which only take place in scenario 1. By contrast, PCOP was higher in scenario 2, since the anisole consumption was higher.

4. Conclusions

Both OFMSW and fruit waste are generated in large quantities worldwide. Their sugar-rich composition makes them suitable raw materials for the production of high-value compounds. Consequently, any development aimed at promoting their re-entry into the value chain is an advance towards sustainability. Based on this premise, in previous works a process was designed and validated for the production of P(3HB) from these organic wastes. In the present work, a techno-economic simulation of this process was carried out to evaluate its viability. The feasibility indicators such as gross and net profit, gross margin, ROI and NPV showed that the

sole production of biopolymers was not feasible. The comparison of the two proposed scenarios showed that although the pretreatment and enzymatic hydrolysis steps had a great impact on the cost of the plant, these were not the main cause of the infeasibility of the process. The most relevant cause was the low yield of the fermentation and was due, at least in part, to the enormous heterogeneity of the waste used as feedstock. Actions such as harnessing waste streams, namely undigested OFMSW from the enzymatic hydrolysis and residual biomass from polymer extraction to produce other compounds could help improve the profitability of the process. The successful implementation of this type of process must not only take into account the techno-economic aspects, but also the environmental and social ones. Therefore, it is necessary to consider and evaluate the impact of all the stages of the process, from the extraction of raw materials to the treatment of the waste generated.

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MANUSCRIPT 4: Protein hydrolysate from Organic Fraction of Municipal Solid Waste compost as nitrogen source to produce lactic acid by *Lactobacillus fermentum* ATCC 9338 and *Lactobacillus plantarum* NCIMB 8826

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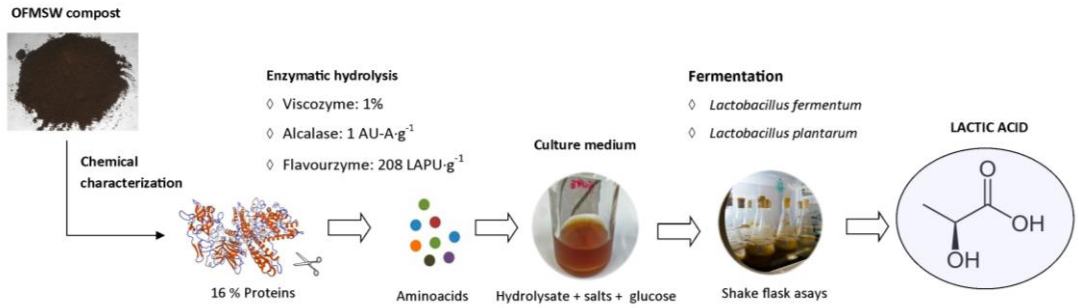
Highlights

- First study on the use of compost as N source for lactic acid production.
- A remarkable recovery of amino-acids (76%) was achieved after enzymatic hydrolysis.
- In a medium with hydrolysate, both strains produced around 10 g·L-1 of lactic acid.
- This process avoids the compost overproduction and promotes the circular economy.

Abstract

In this work a strategy for obtaining free amino-acids concentrate from an organic fraction of municipal solid waste compost and its use as a nitrogen source for lactic acid production, a compound widely used in different industries, using *L.fermentum*ATCC 9338 and *L.plantarum* NCIMB 8826 strains is described. Enzymatic digestion is based on the combined action of endoprotease Alcalase 1.5 MG and exoprotease Flavourzyme 500 MG. The highest degree of hydrolysis obtained under the optimal conditions was 41%. The use of glucanase Viscozyme L prior to protein hydrolysis helped to reduce the viscosity of the solution and promote the action of proteases, increasing its hydrolysis degree by 76 %. The hydrolysate contained all 21 amino-acids, making it ideal for lactic acid bacteria growth. During shake flask cultivations the culture media was complemented with glucose as carbon source. Finally, with the hydrolysate, a maximum lactic acid concentration of 9.0 ± 0.2 $g \cdot L^{-1}$ and 11.1 ± 0.1 $g \cdot L^{-1}$ for *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB 8826 respectively was obtained after 27 h. The innovation of the approach lies in exploiting the overproduction of compost for the production of lactic acid.

Graphical abstract



Keywords

Organic fraction municipal solid waste; Compost; Enzymatic hydrolysis; Protein hydrolysate; Lactic acid

Nomenclature

AU, Anson units; CDW, cell dry weight; DH, degree of hydrolysis; FBG, Fungal Beta-Glucanase units; FLD, fluorescence detector; FMOC, 9-fluorenylmethylchloroformate; HPLC, high performance liquid chromatography; LA, lactic acid; LAB, lactic acid bacteria; LAPU, Leucine Aminopeptidase units; MBT mechanical biological treatment; MRS, Man Rogosa Sharpe; MSW, municipal solid waste; NT, total nitrogen; OD, optical density; OFMSW, organic fraction of municipal solid waste; OPA, o-phtalaldehyde-3-mercaptopropionic acid; PLA, poly(lactic acid); RID, refraction index detector; YE, yeast extract.

1. Introduction

In recent years global municipal solid waste (MSW) production has notably increased and it does not seem that there will be a tangible reduction in the short term. Specifically, it is estimated that 270 million of tons will be generated in Europe in 2019. Approximately 36 % of MSW takes the form of its organic fraction (OFMSW), which is mainly made up of biodegradable materials (e.g., cellulose, hemicellulose, sugars, starch, protein and minerals) [1, 2, 3]. Nowadays the principal treatment

methods are land-filling, incineration, composting, Mechanical Biological Treatment (MBT) and biogas production [2, 4, 5, 6]. Despite efforts to manage it, the decomposition of OFMSW may generate huge environmental problems, including methane emissions, leachates, ecosystem alterations and animal diseases [3, 7, 8]. In this context, composting is a good way to stabilize organic matter. During its production process soil bacteria decompose and break down the biomass, which produces large amounts of heat and water vapor. These conditions favor the development of beneficial microorganisms, to the detriment of pathogens, and help to achieve a stable final product. This technology fits very well into the circular economy strategy and complies with current policies.

European policies are firmly directed at encouraging prevention, reduction and adding value. The latest milestonehas been the waste framework directive [9], which was amended by the European Parliament and European Council [10], where a waste management hierarchy is proposed. In addition, as part of its continuous efforts to improve sustainability and recovery, in 2018 the European Commission adopted several initiatives outlined in the Circular Economy Action Plan [11].

Composting is a well-established process for recycling organic wastes into useful products [12]. In addition, it has several benefits for the environment, such as reducing greenhouse gas emissions, reducing soil erosion, avoiding leachates, increasing water retention capacity and providing nutrients for crops [13, 14, 15]. However, composting processes can lead to odour and bioaerosol generation or accumulation of heavy metals which could have negative health effects [16]. The implementation of suitable measures such as separate collection from municipal bio-waste can help to reduce pollutant contamination of waste and thus improve compost quality, greatly increasing its economic viability [17]. To date no unified criteria for compost from municipal bio-waste in Europe exist, which means that in many cases countries have difficulties exporting and distributing the product [18]. While high quality compost is usually highly appreciated, in other cases composting plants are unable to sell it, causing its overproduction [19]. This is the case of the Basque Country where the scarcity of agricultural soils, the significant surpluses of

livestock wastes together with the negative image of the product reduces its market viability [20].

Taking this scenario into account, it is necessary to find a solution for compost overproduction. It is known that organic nitrogen is the principal form of nitrogen in compost and mainly consists of protein-like compounds [21, 22]. Hydrolysates from these proteins can be useful as a nitrogen source to cultivate microorganisms and produce a wide range of chemical building blocks [23, 24, 25].

Lactic acid (LA), for example, is an organic compound with a high commercial value due to its broad range of applications, such as in food, pharmaceutical, cosmetic, and chemical industries [26]. Its annual world production is about 259,000 metric tons, and approximately 82 % is used by the food industry. Recently, due to the boom in bio-plastics such as poly(lactic acid) (PLA), it is estimated that the lactic acid market will be valued at USD $3.82 billion by 2020 [27, 28].

LA can be produced chemically or biotechnologically [29]. If it is generated through fermentation the isomer produced is almost exclusively L-lactic acid, which permits synthesis of polymers with a higher grade of crystallinity [30].

The fermentative production of LA usually requires large quantities of nitrogen and carbon, whose usual sources are quite expensive. For this reason, this study has developed a process to produce a nitrogen rich concentrate from OFMSW compost. The method consists of enzymatic hydrolysis of proteins. Furthermore, it evaluates the possibility of using the concentrate as a culture media to cultivate lactic acid bacteria (*L. Fermentum* ATCC 9338 and *L. Plantarum* NCIMB 8826) at a laboratory scale in order to produce lactic acid [31,32,33,34,35].

2. Materials and methods

2.1. Chemicals and MSW compost biomass

Free amino acid standards and reagents for amino acid analysis were obtained from Agilent Technologies (Agilent Technologies Spain S.L., Madrid). The chemicals

used for analytical methods were potassium dihydrogen phosphate, sodium hydrogen phosphate, methanol, acetonitrile, glucose and lactic acid obtained from Sigma-Aldrich (Steinheim, Germany). Also, sodium acetate, triammonium citrate, magnesium sulfate, manganese (II) sulfate and potassium hydroxide from Panreac (Barcelona, Spain) were used. Reagents for culture media preparation Man Rogosa Sharpe (MRS) broth and salts were obtained from Sigma-Aldrich (Steinheim, Germany).

Municipal solid waste compost used in this study was collected from EPELE composting plant (Gipuzkoa, Spain). Before hydrolysis the biomass was lyophilised and screened using a mesh (Endecotts, England) with a 2 mm particle size. These residues contained (w/w) moisture (41 %), ash (18 %), proteins (16 %), crude fiber (12 %), others (11 %), soluble sugars (1 %) and fats (1 %).

2.2. Enzymes

The enzymes used were Alcalase1.5 MG (endoprotease from *Bacillus licheniformis*, 1.5 (Anson units (AU-A)·g^{-1}), Flavourzyme500MG (exoprotease and endoprotease complex from *Aspergilus oryzae*, 500 (Leucine Aminopeptidase units (LAPU)·g^{-1}) and Viscozyme L (betaglucanase-cellulase-xylanase from *Aspergillus aculeatus,* 100 (Fungal Beta-Glucanase units (FBG)·g^{-1}) were donated by Novozymes S.A (Madrid, Spain). The latter was used to reduce the viscosity of the reaction media and thus improve hydrolysis yield.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis optimization was carried out in 500 mL conical flasks containing 14.4 % (w/v) biomass suspension in distilled water. To control agitation and temperature, an Infors HT Ecotron (Switzerland) incubator was used. First, the glucanase Viscozyme L (1 %; 50 °C; pH 4.5) was added to reduce the viscosity of the solution and to promote the action of proteases. Thirty minutes later its pH was adjusted manually to 7 by adding NaOH. Afterwards Alcalase 1.5 MG (1 AU-A·g^{-1}; 50 °C; pH 7), an endoprotease, was used and finally after 150 minutes the exoprotease

Flavourzyme 500 MG (208 LAPU·g^{-1}; 50 °C; pH 7) was added continuing with hydrolysis until 270 min. Throughout hydrolysis, 1 mL samples were taken to monitor the process. The hydrolysis reaction was stopped by heating in an autoclave at 121 °C for 20 minutes. The free amino-acid rich hydrolysate was recovered by centrifugation at 10,000 rpm (Beckman Coulter Avanti J-26 XP; Brea, CA, USA) and the final concentrate was cleaned using an 11 μm paper filter (Whatman, NO.1; Chiltern, UK). Finally, the broth obtained was autoclaved before its use in carrying out some tests of growth with lactobacillus.

2.4. Microorganisms, culture media and inoculum preparation

***Table 1**. Culture medium composition.*

Composition	**Control Medium**	**Hydrolysate + salts**
Glucose (g·L^{-1})	20.0	20.0
Yeast extract (g·L^{-1})	4.00	-
Salts (g·L^{-1})	9.25	9.25
OFMSW compost hydrolysate (mL·L^{-1})	-	400
NT (g·L^{-1})[a]	0.40	0.40

[a]**NT**: Total nitrogen

Two strains of lactic acid bacteria (LAB), *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB8826 were stored in Man Rogosa and Sharpe (MRS) agar stabs at 4 °C. After two successive transfers of the test organisms in MRS broth at 37 °C for 12–15 h, cultures in exponential phase were reinoculated into MRS broth and grown for 16 h at 37 °C. These cultures were used as inoculum. The control medium (MRS) for flask cultures was (per litre): K_2HPO_4, 2.0 g; sodium acetate·$3H_2O$, 5 g; triammonium citrate, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.05 g; yeast extract, 4 g and the medium made with OFMSW compost hydrolysate was (per litre): K_2HPO_4, 2.0 g; sodium acetate·$3H_2O$, 5 g; triammonium citrate, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.05 g. The pH was adjusted to 6.5 with KOH (2 M) and the carbon source for both the control medium and the medium made with hydrolysate was

glucose (20 $g \cdot L^{-1}$). Table 1 summarizes and compares the composition of both mediums.

2.5. Culture conditions

Shaking flask assays were carried out to determine and compare the growth and lactic acid production of *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB 8826 between the standard medium and the hydrolysate. The tests were performed in 500 mL conical flasks containing 100 mL of liquid phase. The inoculum quantity was 2 % (v/v) of an active culture of the microorganism, which was allowed to ferment at 37 °C for 27 h. These assays were performed in duplicate and their average value was taken.

2.6. Analytical methods

Cellular growth was monitored by measuring the optical density (OD) of samples at 600 nm in a spectrophotometer (Shimadzu UV-2401PC; Kyoto, Japan). Cell dry weight (CDW) was determined after drying pellets of the samples (1.2 mL) in an oven (Selecta 200; Barcelona, Spain) at 60°C until constant weight.

The OFMSW compost dry matter was determined after weighing a certain amount of biomass and drying in an air ventilated oven, at 105 °C, for 24 hours until constant weight. Ash content was obtained by combusting the dry matter in a muffle furnace (Thermo-Scientific; Asheville, NC, USA) at 600 °C.

Sugars and lactic acid in cultures and hydrolysis samples were analyzed by high performance liquid chromatography (HPLC) (Agilent Technologies 1260 Infinity II; Madrid, Spain) equipped with a Hi-Plex H (300 mm x 7.7 mm, Agilent) column, an auto sampler (Agilent 1260 Vial-sampler G7129A) and an Agilent 1260 RID G7162A refraction index detector. The column work temperature was 65 °C. 2.4 µL of sample volume was added on a described column and eluted isocratically with a flow rate of 0.5 $mL \cdot min^{-1}$ of a 5 mM H_2SO_4 solution. Each analysis was carried out in duplicate;

peak identification and quantification were achieved using standards of glucose and lactic acid. The mean concentration of two analyses is presented.

Amino-acids were analyzed by HPLC (Agilent Technologies 1260 Infinity II) system equipped with an auto sampler (Agilent 1260 Vial-sampler G7129A) and a fluorescence detector (Agilent 1260 FLD Spectra). Precolumn derivatization was automated with o-phtalaldehyde-3-mercaptopropionic acid (OPA; Agilent Technologies Spain S.L., Madrid, Spain) for primary and 9-fluorenylmethylchloroformate (FMOC; Agilent Technologies Spain S.L., Madrid, Spain) for secondary amino-acids [36]. A 100 mm Advanced Bio AAA column (4.6 mm; 2.7 μm, Agilent) was used for its separation. The optimal operating temperature and the injection volume were 40 °C and 1 μL respectively. Phosphate buffer (pH 7.8, A) and methanol/acetonitrile/water (45:45:10, B) were used as mobile phase at a flow rate of 1.5 $mL \cdot min^{-1}$. The elution gradient was as follows: starting with 2 % (B) it was maintained for 0.35 min, then increased to 57 % in 13.05 min, to 100 % in 0.1 min and it remained for 2.2 min. Finally, it decreased to 2 % in 0.1 min where it was maintained for 2.2 min to re-equilibrate the column before the next run. The excitation wavelengths of fluorescence were set at 340 nm, and the emission wavelengths were acquired over the range of 365-495 nm with intervals of 5 nm. Norvaline and sarcosine (Agilent TechnologiesSpain SL, Madrid, Spain) were used as internal standards for primary and secondary amino-acids. Standards were used for peak identification and quantification amino-acids.

Fiber (cellulose, hemicellulose and lignin) analyses were carried out using an ANKOM 2000 fiber analyzer (Macedon, NY, USA) [37].

Total carbon, nitrogen and hydrogen were determined using an elemental analyzer (LECO CHNS-932; Michigan, USA). Protein content (%) in the biomass samples was calculated as 6.25 times the total percent nitrogen in the sample [38]. The fats were extracted according to the Folch, Less and Stanley method (1957) and determined by gravimetry.

2.7. Statistical analysis

Results are presented as means value ± standard deviation. A one-way analysis of variance (ANOVA) and 95 % confidence level were used to see the differences between means of each group. Data with P-value < 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Enzymatic hydrolysis

OFMSW compost contains a wide variety of nutrients that make it ideal for growing plants, but its low economic value requires one to seek alternative added-value solutions for its use [39, 19]. One route may be to tap into its high content in proteins (16 % (w/w)) in order to produce free-amino-acids concentrates through enzymatic hydrolysis and use this latter in lactic acid fermentation. With reference to enzymatic hydrolysis, searching for the suitable enzymes and optimizing the conditions is vital. Although the range of commercial enzymes is very wide, their application usually focuses on conversions under recommended conditions and their use in new substrates requires application test trials. The first conditions used in the hydrolysis were chosen after bibliographic revision. When the degree of hydrolysis (DH) reached was not sufficient, parameter optimizations such as enzyme quantity, hydrolysis time and influence of biomass concentration were carried out. The enzymatic process was undertaken under the recommended conditions of temperature (50 °C) and pH (7). Initially, the endoprotease Alcalase 1.5 MG was added to the reaction mixture; as a result, amino-acid concentration increased progressively until after 120 min when it reached constant concentration. Afterwards, the exoprotease Flavourzyme 500 MG was added to finish hydrolysis [40, 41]. Enzyme quantity optimization was started with 0.2 AU-A·g^{-1} Alcalase 1.5 MG and 41.6 LAPU·g^{-1} Flavourzyme 500 MG. Then the enzyme concentration was increased until maximum hydrolysis yield was reached. The optimum quantities of both Alcalase 1.5 MG and Flavourzyme 500 MG were 1 AU-A·g^{-1} and 208 LAPU·g^{-1} respectively. The data showed that using large amounts of enzyme did not result in increased DH (Fig. 1).

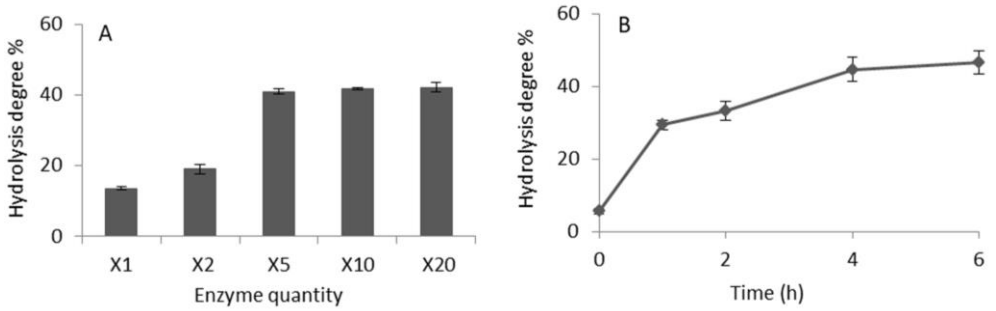


Fig.1: Hydrolysis degree variation with the enzyme quantity (X1: 0.2 AU-A·g^{-1} substrate (Alcalase 1.5 MG) and 41.6 LAPU·g^{-1} substrate (Flavourzyme 500 MG); 6h; pH 7; 50 °C). X2, X5, X10 and X20 corresponded to the different enzyme loads used and were two, five, ten and twenty times higher than X1, respectively (A) and time of hydrolysis (X5: 1 AU-A·g^{-1} substrate (Alcalase 1.5 MG); 208 LAPU·g^{-1} substrate (Flavourzyme 500 MG); pH 7; 50 °C) (B). Error bars indicate standard deviation (n=3).

This concentration of enzymes was employed for the study of the subsequent parameters. Next, hydrolysis time and biomass influence were investigated. The reaction time was selected based on the time at which the DH began to be constant. The optimal time for the total hydrolysis process was established at 240 min, 120 min for endoprotease alone, plus 120 min for exoprotease. One of the most relevant parameters in the enzymatic process is the influence of biomass composition and its concentration in the hydrolysis. It is reasonable to assume that the use of high biomass concentration during hydrolysis will lead to a greater quantity of hydrolysed product. However, there are several factors that determine this effect, such as a low mass transfer (rheological effects), an inherent recalcitrance of the substrate, a high enzyme inhibition or peptide degradation. Ideally, one would obtain the greatest amount of amino-acids using the largest concentration of biomass, while the DH remains high. Biomass concentrations ranging from 24 to 228 g·L^{-1} were used for the study of the effect of the biomass quantity. To carry this out, a constant enzyme substrate ratio was maintained and the water volume was varied. Fig. 2 shows the variation of both free amino-acids and DH with the biomass quantity.

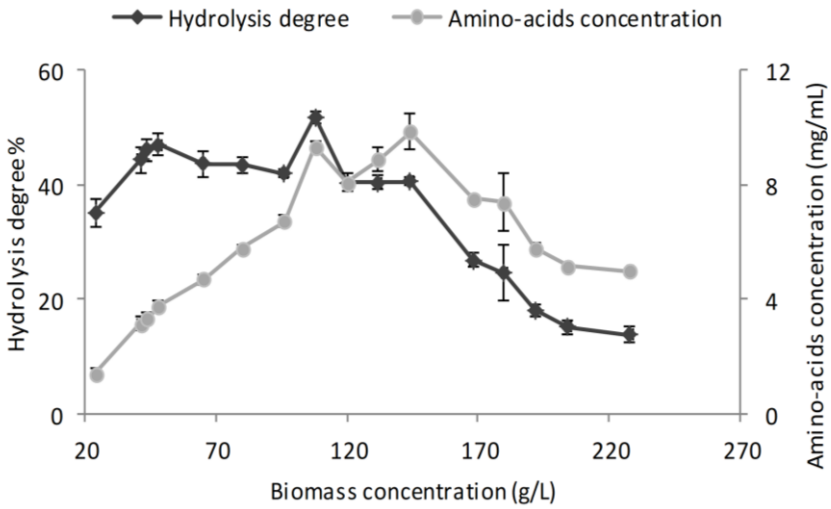


Fig. 2: Influence of OFMSW compost biomass concentration in the hydrolysis degree and free-aminoacid concentration after enzymatic hydrolysis under standard conditions (Alcalase 1.5 MG (1 AU-A·g^{-1} substrate); Flavourzyme 500 MG (208 LAPU·g^{-1} substrate); 4.5 h; pH 7; 50 °C). Error bars indicate standard deviation (n=3).

At low substrate levels (24 g·L^{-1}), the amino-acid concentration and therefore the DH were small. When substrate quantity is increased this usually results in an increase in the amino-acids released. However, if the increase is too great, substrate inhibition can occur, resulting in a lower hydrolysis yield. The optimum substrate concentration for high release of free amino-acids and high DH was 144 g·L^{-1}. At this point, the maximum DH obtained had been around 41 %. Therefore, further improvement in DH by reducing viscosity of the media by applying enzymes and thereby improving amino-acid release was contemplated. Several authors suggest that adding the glucanase Viscozyme L to the mixture can break up the polysaccharides and make proteins more accessible [42]. The results obtained indicate that using Viscozyme L as a pre-treatment before the protein hydrolysis increases the yield of the process from 42 % to 76 % (Fig. 3). The combined action of glucanase and of the two proteases may well have contributed substantially to this significant increase. There are some reports on the use of other wastes such as defatted microalgae biomass, defatted soy flour and fish flour for amino-acids production by means of similar processes [42, 43, 44]. However, their

hydrolysis yields were substantially lower (from 25 % to 59 %) than those obtained in this study (76 %).

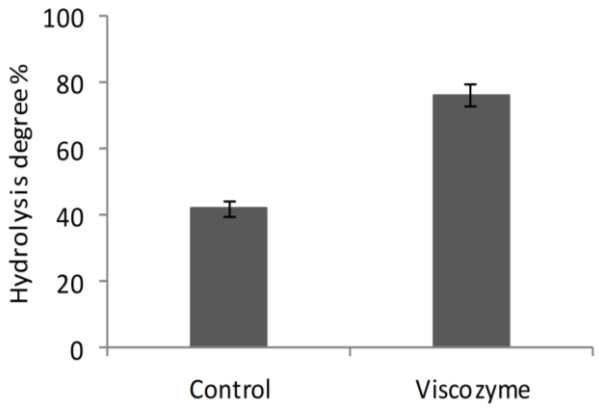


Fig. 3: Influence of 1 % of Viscozyme L in the hydrolysis degree under standard conditions (1 AU-A·g^{-1} substrate (Alcalase 1.5 MG); 208 LAPU·g^{-1} substrate (Flavourzyme 500 MG); 270 min; pH 7; 50 °C; 144 g·L^{-1} (biomass concentration)). Control had no Viscozyme L addition. Error bars indicate standard deviation (n=3).

Following a circular economy strategy, the final product obtained through the process described could be used to obtain new products with economic interest, while at the same time minimizing discharge. In fact, there are several studies that explain in detail the use of protein hydrolysates as nitrogen source to cultivate microorganisms [45, 46, 47]. In this respect the investigation described here achieved promising results.

3.2. Amino-acid characterization

The free amino-acid profile of OFMSW compost hydrolysate is shown in table 2. It contained a comprehensive distribution of amino-acids.

Table 2. Free amino-acid composition of compost hydrolysate and commercial yeast extract.

	Compost hydrolysate[a]	**Yeast extract (YE)**	
	g/100g hydrolysate	Free (g/100g YE)	Total (g/100g YE)
Aspartic acid	0.12 ± 0.02	0.15	6.50
Glutamic acid	0.12 ± 0.02	0.64	11.5
Aspargine	0.061 ± 0.003	-	-
Serine	0.035 ± 0.007	0.19	2.40
Glutamine	0.03 ± 0.01	-	-
Histidine	0.04 ± 0.02	0.09	1.40
Glycine	0.17 ± 0.02	0.12	3.00
Threonine	0.10 ± 0.01	0.16	2.60
Arginine	0.026 ± 0.001	0.13	3.20
Alanine	0.28 ± 0.02	0.35	4.80
Tyrosine	0.10 ± 0.03	0.03	1.10
Cysteine	0.10 ± 0.03	0.02	-
Valine	0.20 ± 0.07	0.31	4.10
Methionine	0.04 ± 0.01	0.08	0.90
Tryptophan	0.03 ± 0.01	0.15	0.09
Phenylalanine	0.07 ± 0.01	0.21	2.60
Isoleucine	0.09 ± 0.02	0.23	3.10
Leucine	0.15 ± 0.02	0.43	4.20
Lysine	0.08 ± 0.01	0.14	4.60
Proline	0.07 ± 0.01	0.09	2.30
Total	1.90	3.52	58.39

[a]Data are mean ± standard deviation.

By comparing the profile obtained with the profile of yeast extract (YE) (Yeast extract 7184; Neogen Corporation (USA)) (Yeast extract A-1202; Solabia Group (France)) it is observed that the hydrolysate has a wider spectrum of amino-acids. This discrepancy could be ascribed to the fact that most amino nitrogen in yeast extract takes the form of protein.

Furthermore, alanine, valine and glycine were the major amino-acids in the hydrolysate, representing as much as 34 % of the total. Moreover, the sum of non-essential amino-acids was higher than the essential ones, but the presence of the latter was remarkable. It is important because lactic acid bacteria cannot (in most cases) synthesize essential amino-acids (such as valine, leucine, isoleucine, methionine, glutamine and histidine), as they require an exogenous source for this purpose [48, 49, 50].

Other studies applying similar processes but using agri-food residues as raw material also obtained a complete distribution of amino-acids [51, 52, 53]. Due to the lower protein concentration of compost compared with this raw material the amount of released amino-acid is lower. Nevertheless, the product obtained has the necessary qualities for its use as a nutritive medium to cultivate lactobacillus.

3.3. Shaking flask cultivations

Both *L. fermentum* ATCC 9338 and *L. plantarum* NCIMB 8826 are heterofermentative bacteria and produce the lactic acid racemic mixture. However, they demonstrate great ability to metabolize several monosaccharides (glucose, xylose, arabinose, and galactose, among others) simultaneously [54, 55]. This is a positive factor, since it would allow one to complement the designed culture media with carbon-rich waste concentrates. The development of gene manipulation technologies has allowed strains capable of producing a single isomer of lactic acid to be obtained [56]. Combining this capacity with the ability to use different monosaccharides not only improves the process but also increase the number of exploitable wastes. There are several studies in which protein rich waste hydrolysates were used to synthetize lactic acid via bacterial fermentations. For

example, Safari et al. used *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrukii, Lactobacillus plantarum*, *Lactococcus lactis* and *Lactobacillus sakei* on yellowfin tuna (*Thunnus albacares*) hydrolysate [57], Yuan Li et al. and Zheng Li et al. used *Sporolactobacillus laevolacticus* DSM442 on cottonseed hydrolysate and *Lactobacillus casei* LA-04-1 on soybean meal hydrolysate respectively [58, 59]. Nevertheless, this is the first study of lactic acid production from OFMSW compost by of *L. plantarum* NCIMB 8826 and *L. fermentum* ATCC 9338.

Shaking flask assays were designed to establish cell growth, glucose consumption and lactic acid production.

Figure 4A and B shows the profile of fermentation of *L. Fermentum* ATCC 9338 and *L. Plantarum* NCIMB 8826 respectively, using the MRS medium. Both strains consumed the glucose at around 22 h and their growth was similar, but the lactic acid production of L. *Plantarum* NCIMB 8826 (15.5 $g \cdot L^{-1} \pm 0.5$) was a little higher than that of *L. fermentum* ATCC 9338 (13.2 $g \cdot L^{-1} \pm 0.3$). The conversion yield of glucose to lactic acid was 1.2 times higher with *L. Plantarum* NCIMB 8826 (0.86 $g \cdot g^{-1} \pm 0.03$).

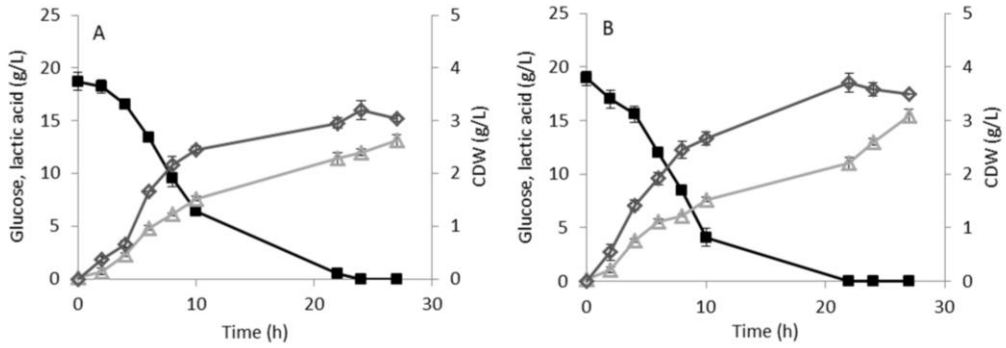


Fig. 4: Growth and lactic acid production of L. fermentum ATCC 9338 (A) and of L. plantarum NCIMB 8826 (B) with MRS medium. CDW (open diamond), lactic acid (open triangle) and glucose (closed square). Error bars indicate standard deviation (n=2).

When the OFMSW compost hydrolysate was used as nitrogen source, slower glucose consumption was noted (figure 5A and B), which meant a delay in the cell

growth for both strains. The yield of lactic acid with hydrolysate was 0.59 $g \cdot g^{-1} \pm 0.01$ and 0.64 $g \cdot g^{-1} \pm 0.03$, and lactic acid production was $9.0 \pm 0.2 g \cdot L^{-1}$and $11.1 \pm 0.1 g \cdot L^{-1}$ for *L. fermentum* ATCC9338 and *L. plantarum* NCIMB 8826 respectively.

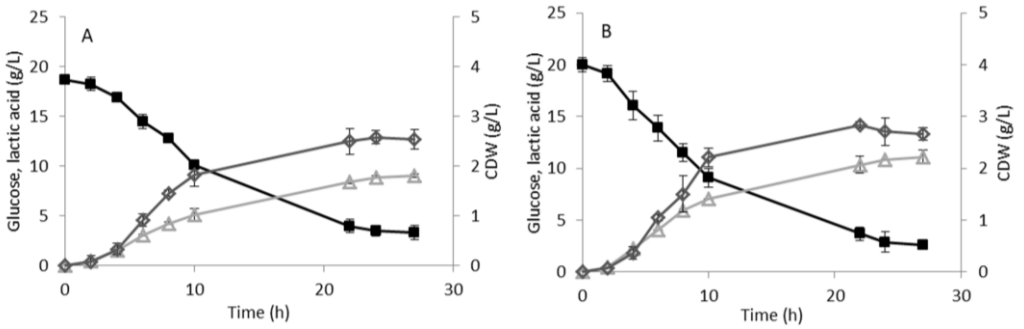


Fig. 5: Growth and lactic acid production of L. fermentum ATCC 9338 (A) and of L. plantarum NCIMB 8826 (B) with medium made with hydrolysate. CDW (open diamond), lactic acid (open triangle) and glucose (closed square). Error bars indicate standard deviation (n=2).

Despite the bacteria being heterofermentative, the yields obtained were high. This could indicate that sugars were not metabolized through the phosphoketolase pathway. According to this, only 1 mol of lactic acid should be produced from 1 mol of glucose [60]. This result is in line with that obtained by Okano et al., who reported a D-lactic acid production of 73.2 g/L with a yield of 0.85 g/g glucose by a metabolically engineered strain (L. plantarum NCIMB 8826) to produce only one isomer [56]. Another study in which this genetically modified strain was also used, again showed a high concentration (61.4 g / L) and yield (0.77 g / g glucose) [61]. In this work the experiments were carried out at shake flask scale using low concentrations of glucose and with no on-line monitoring and control capabilities. It is expected that a scale-up using bioreactors will improve the control of the fermentation parameters and thus the yield [62, 23].

On the other hand, the difference in growth and lactic acid production between MRS medium and hydrolysate may be explained by the fact that MRS medium contains yeast extract which, apart from being an organic nitrogen source, contains a wide range of microelements, vitamins and growth factors. These compounds may contribute not only to high biomass but also to a high lactic acid metabolism level

[53]. Another possible cause could be the presence of some kind of inhibitor in the hydrolysate.

YE is the source of nitrogen most used at a laboratory scale, but its high economic cost makes it unfeasible for industrial use [63, 64]. According to some authors, nitrogen source and yeast extract represents 38 % of the total cost during lactic acid production [26, 65].

The results obtained are particularly interesting because they open the door to continually improving the process on greater scales. Moreover, this method is not only a low-cost way to produce lactic acid, but is also a way to reduce the amount of organic waste. In this line, carbon-rich waste could be combined with this nitrogen source and thereby reduce production costs.

4. Conclusions

In specific cases, such as that of Basque Country, the accumulation of compost biomass due to overproduction represents a real problem. This method is a useful solution to add value to OFMSW compost as a nitrogen source for fermentation processes, producing for example with *L. fermentum* ATCC 9338 and *L.plantarum* NCIMB 8826. The use of the glucanase Viscozyme L in the enzymatic hydrolysis was fundamental in reducing the viscosity of the medium and increase amino-acid release. The culture media made with compost hydrolysate had to be complemented with other nutrients in order to satisfy the nutritional requirements of the microorganisms. Finally, to further improve environmental sustainability and to reduce processing costs, it could be combined with carbon-rich residues such as agricultural waste, thereby contributing to the implementation of a circular bio-economy.

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ANNEX 2: PUBLICATIONS RELATED TO THE PRESENT DOCTORAL THESIS

MANUSCRIPT 5: Upgrading end-of-line residues of the red seaweed *Gelidium sesquipedale* to polyhydroxyalkanoates using *Halomonas boliviensis*

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Upgrading end-of-line residues of the red seaweed *Gelidium sesquipedale* to polyhydroxyalkanoates using *Halomonas boliviensis*

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Abstract

Agar extraction from *Gelidium* and *Gracilaria* red seaweed species produces hundred thousand ton of carbohydrate-rich residues annually. *Gelidium sesquipedale* waste biomass obtained after agar extraction, still contained 44.2 % w/w total carbohydrates (dry-weight basis). These residues were biologically upgraded to poly-3-hydroxybutyrate (P3HB) after saccharification of their carbohydrate fraction to simple sugars. A combined hydrolysis treatment using sulfamic acid followed by enzymatic hydrolysis with cellulases produced a glucose-rich hydrolysate with a negligible content of inhibitors. With this treatment a sugar yield of circa 30 % (g glucose/g biomass) was attained. The algal hydrolysates were assessed as carbon source for the production of P3HB by the halotolerant bacteria *Halomonas boliviensis*. A cell concentration of 8.3 $g \cdot L^{-1}$ containing 41 % (w/w) of polymer and a yield ($Y_{P/S}$) of 0.16 $g_{polymer}/g_{glucose}$ were attained in shake flask assays. In this work, cellulose-rich seaweed waste was shown to be an upgradable, sustainable source of carbohydrate