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TESIS DOCTORAL:

PROCESSES FOR THE VALORIZATION OF MICROALGAL BIOMASS CULTIVATED IN PIGGERY WASTEWATER

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RESUMEN

La generación de aguas residuales de purines se ha convertido en un problema medioambiental que debe resolverse gestionando estos residuos mediante un tratamiento adecuado. Una tecnología prometedora es el uso de fotobiorreactores donde microalgas y bacterias actúan simbióticamente para eliminar los nutrientes, lo que permite reducir costes en el tratamiento de las aguas residuales y en la producción de biomasa algal al utilizar las aguas residuales como medio de cultivo. En este tratamiento se genera una biomasa valorizable que puede utilizarse para producir diversos componentes y bioproductos. Sin embargo, la mayoría de los componentes de las algas, como proteínas y carbohidratos, se encuentran dentro de una pared celular que debe romperse para poder extraerlos, con una baja degradación de los componentes solubilizados y teniendo en cuenta la presencia de contaminantes. Así, esta tesis se centra en la valorización de la biomasa algal cultivada en aguas residuales de purines mediante diferentes tecnologías. Así mismo, se ha estudiado la viabilidad económica y ambiental de algunos procesos de valorización propuestos.

Los análisis tecno-económicos y medioambientales demuestran que se pueden obtener productos agrícolas de forma sostenible a partir de biomasa algal cultivada en aguas residuales de purines. El método de cosechado de la biomasa algal fue el punto crítico del proceso, obteniéndose un bioestimulante 4.5 veces más concentrado con centrífugas que con membranas. Sin embargo, el uso de centrífugas conllevó un gran gasto debido al alto coste de este tipo de equipo y sus requerimientos de electricidad, resultando un coste final de producción de bioestimulante de 343 €/m^3 frente a 66 €/m^3 usando membranas. Comparando el tratamiento de 1ha de cultivo, el coste del bioestimulante producido con centrífuga o membrana fue inferior al producto comercial (22.1% y 48.1% respectivamente). El uso de membranas para el cosechado de la biomasa permitió plantas económicamente viables capaces de operar a menor capacidad y distribuir bioestimulantes a mayores distancias que utilizando centrífugas. El análisis ciclo de vida de la producción de bioestimulantes mostró un impacto ambiental un 30% menor utilizando membranas (impacto en el calentamiento global (GW) de 217 kg CO_2 eq/ha) que centrífugas. La elevada cantidad de bioestimulante requerida para los cultivos cuando se utilizó el sistema de membranas, también conllevó un impacto ambiental relevante del transporte del bioestimulante, siendo la membrana más favorable cuando la distancia a los cultivos es inferior a 321 km. Como alternativa, se

evaluó la extracción de biopesticidas antes de la producción del bioestimulante. Los costes de producción de biopesticidas calculados fueron de 0.35 €/L utilizando membranas y de 2.12 €/L con centrífuga, pero con mayores costes de inversión y un proceso más complejo que la producción de sólo bioestimulantes.

La aplicación de una valorización fraccionada de los diferentes componentes de las algas podría proporcionar mayores beneficios. No obstante, la solubilización y recuperación de los componentes de la biomasa requieren la rotura de la pared celular. Se compararon diferentes métodos de extracción y condiciones de operación. La hidrólisis enzimática permite obtener péptidos y monosacáridos en condiciones suaves. El mayor rendimiento de solubilización de carbohidratos (38.5%) se consiguió a partir de biomasa de microalgas-bacterias utilizando Celluclast y un tiempo de hidrólisis de 5 horas. La hidrólisis enzimática con Alcalasa proporcionó la mejor recuperación de péptidos (34%) con tamaños de péptidos bajos (<10 kDa), mientras que se obtuvieron recuperaciones de péptidos bajas (<20%) pero con grandes tamaños de péptidos (hasta 135 kDa) tras la hidrólisis enzimática con Protamex. Comparando la hidrólisis enzimática de biomasa de microalgas-bacterias y de microalgas puras, se obtuvieron menores pérdidas por degradación a partir de biomasa de microalgas-bacterias.

Con el fin de mejorar los rendimientos de extracción aplicando condiciones de hidrólisis suaves, se estudiaron métodos novedosos que combinaban tratamientos físicos y biológicos. El acoplamiento de ultrasonidos e hidrólisis enzimática con Protamex (UAEE) permitió recuperar el 43.6% de las proteínas como péptidos grandes (hasta 135 kDa) y con la mayor pureza (46.7%). Por otro lado, la solubilización de carbohidratos aumentó tras aplicar una extracción enzimática asistida por microondas (MAEE), alcanzando rendimientos del 73% de xilosa, pero también con pérdidas significativas. Sin embargo, estos resultados fueron inferiores a los obtenidos mediante tratamientos químicos en condiciones más severas, por lo que se hace necesaria una mayor optimización.

Para conocer el efecto de los contaminantes en la composición de la biomasa, los rendimientos de extracción y la seguridad de los productos finales, se llevaron a cabo experimentos en una planta piloto alimentada con aguas residuales de purines dopadas con antibióticos veterinarios (sulfadiazina, tetraciclina y ciprofloxacina), y elementos tóxicos (Cu, Zn, As). Ambos tipos de contaminantes disminuyeron el contenido de

glucosa de la biomasa algal hasta en un 42% y aumentaron los contenidos de proteína y xilosa en un 30% y 16% debido al estrés oxidativo, modificando la estructura. Estas variaciones también afectaron a los métodos de extracción, ya que los contaminantes aumentaron la solubilización de proteínas por hidrólisis ácida a 120ºC en un 32%, mientras que redujeron la solubilización de glucosa en un 49% tras hidrólisis alcalina a 120ºC. Las recuperaciones de glucosa y xilosa se redujeron drásticamente en presencia de metales pesados (~100%) debido a la inhibición enzimática tras la hidrólisis enzimática y la EAU con Protamex. Los antibióticos veterinarios aumentaron la solubilización de la xilosa por ultrasonidos (74%), pero también su degradación. Estos métodos suaves dieron lugar a una menor presencia de metales dopados en los hidrolizados finales que los métodos químicos, en los que se encontró más del 60% de los metales dopados en el producto final.

Basándonos en los resultados anteriores, se estudió un proceso de biorrefinería de valorización fraccionada para producir péptidos y polihidroxialcanoatos (PHAs) a partir de biomasa microalgal cultivada en aguas residuales, realizando evaluaciones tecno-económicas y medioambientales. El proceso propuesto resultó económicamente viable, con un valor actual neto (VAN) de ~1.420.000 euros y un período de amortización de 10.6 años. Se necesitó un coste de inversión de ~4.863.000 € relacionado con un coste total del equipo de ~1.654.000 € y un gasto del 65% en centrifugadoras. El coste anual de operación fue de ~619.000 euros, debido al elevado coste de producción de microalgas y a las elevadas necesidades de electricidad y calentamiento. Este proceso alcanzó un GW de 294 kg de CO_2 eq/m^3 de biomasa con la electricidad como principal contribuyente. Este análisis ex-ante permitió identificar los puntos críticos que deben optimizarse en futuras investigaciones para mejorar la viabilidad económica y medioambiental. Así, el VAN aumenta hasta un 141% (si se reduce el número de centrifugadoras), un 91% (si el coste de producción de la biomasa disminuye hasta 0.7 €/kg_{DCW}), un 84% (si el contenido de PHAs en los microorganismos aumenta hasta el 70%) y un 60% (si se reducen los requisitos energéticos en la extracción por ultrasonidos). Asimismo, el GW se reduce en un 15% optimizando los requisitos de electricidad de los ultrasonidos.

ABSTRACT

Piggery wastewater generation has become an environmental problem which must be solved by managing the residue through appropriate treatment. One promising technology is the use of photobioreactors where microalgae and bacteria act symbiotically to remove nutrients, which allows to reduce costs in wastewater treatment and in microalgae production by using the wastewater as culture media. In this treatment process, a valuable biomass is generated that can be used to produce several components and bioproducts. However, most of the algal components such as proteins and carbohydrates are found within a cell wall that should be disrupted to extract them by trying to cause low degradation of the solubilized components and considering the presence of pollutants. So, this thesis focusses on the valorization of the algal biomass grown on piggery wastewater by different technologies. Likewise, the economic and environmental viability of some proposed valorization processes was studied.

Techno-economic and environmental analysis show that agricultural products can be sustainably produced from algal biomass grown in piggery wastewater. The method of harvesting the algal biomass resulted the critical point of the process, obtaining 4.5 times more concentrated biostimulant with centrifugation than using membranes. However, the use of centrifuges led to a great expense due to the high cost of this equipment and its electricity requirements, resulting a final biostimulant production cost of 343 €/m^3 vs 66 €/m^3 using membranes. Comparing the treatment of 1ha of crop, the cost of the biostimulant produced with centrifuge or membrane was lower than commercial product (22.1% and 48.1% respectively). The use of membranes for biomass harvesting allowed the construction of economically viable plants capable of operating at lower capacity and distributing biostimulants over greater distances than using centrifuges. A life cycle assessment of biostimulant production showed a 30% lower environmental impact by using membranes (global warming impact (GW) of 217 kg CO_2 eq/ha) instead of centrifugation. The high amount of biostimulant required for crops when membrane was used, also led to a relevant environmental impact of biostimulant transportation, being membrane more favourable when distance is lower than 321 km. As alternative, the extraction of biopesticides before biostimulant production was evaluated. The calculated biopesticides production costs were 0.35 €/L using membranes and 2.12€/L with centrifuge, but with higher inversion costs and more complex process than producing only biostimulants.

The application of a fractional valorization of different algal components could provide greater benefits. Nevertheless, the solubilization and recovery of biomass components require the disruption of the cell wall. Different extraction methods and operational conditions were compared. Enzymatic hydrolysis allows to obtain peptides and monosaccharides at mild conditions. The highest carbohydrate solubilization yield (38.5%) was achieved from microalgae-bacteria biomass using Celluclast and 5 hours hydrolysis time. Enzymatic hydrolysis with Alcalase provided the best peptide recovery (34%) with low peptide sizes (<10 kDa), while low peptide recoveries (<20%) but large peptide sizes (up to 135 kDa) were obtained after enzymatic hydrolysis with Protamex. Comparing the enzymatic hydrolysis of microalgae-bacteria biomass and pure microalgae, lower losses by degradation were obtained from microalgae-bacteria biomass.

Trying to increase the extraction yields while applying mild hydrolysis conditions, novel methods combining physical and biological treatments were studied. The coupling of ultrasounds and enzymatic hydrolysis with Protamex (UAEE) allowed to recover 43.6% of the proteins as large peptides (up to 135 kDa) with the highest purity (46.7%). On the other hand, carbohydrate solubilization increased after applying a microwave-assisted enzymatic extraction (MAEE), achieving yields of 73% of xylose, but also with significant losses. However, these results were lower than those achieved through chemical treatments at harsh conditions, making further optimization necessary.

To know the effect of pollutants in biomass composition, extraction yields and the safety of the final products, pilot plant experiments feeding piggery wastewater doped with veterinary antibiotics (sulfadiazine, tetracycline and, ciprofloxacin), and toxic elements (Cu, Zn, As) were carried out. Both types of pollutants decreased the glucose content of algal biomass by up to 42% and increased protein and xylose contents by 30% and 16% due to the oxidative stress, also modifying the structure. These variations also affected extraction methods since pollutants increased protein solubilization by acid hydrolysis at 120ºC by 32% while reduced glucose solubilization by 49% after alkaline hydrolysis at 120ºC. Glucose and xylose recoveries were drastically reduced in presence of heavy metals (~100%) due to enzyme inhibition after enzymatic hydrolysis and UAEE with Protamex. Veterinary antibiotics increased xylose solubilization by ultrasonication (74%), but also its degradation. These mild methods resulted in lower

presence of doped metals in final hydrolyzates than chemical methods, where >60% of doped metals were found in the final product.

Based on previous results, a biorefinery process of fractional valorization to produce peptides and polyhydroxyalkanoates (PHAs) from microalgal biomass grown in wastewater was studied, performing techno-economic and environmental assessments. The proposed process was economically feasible with a net present value (NPV) of ~1,420,000 € and payback period of 10.6 years. An investment cost of ~4,863,000 € was needed related to a total equipment cost of ~1,654,000 € with 65% spending on centrifuges. Annual operation cost was ~619,000 € related to the high microalgae production cost and high electricity and heating requirements. This process achieved a GW of 294 kg CO_2 eq/m^3 biomass with electricity as the main contributor. This ex-ante analysis allowed to identify hotspots which must be optimized in future research to enhance economic and environmental feasibility. Therefore, increasing the NPV up to 141% (if the number of centrifuges was reduced), 91% (if biomass production cost diminishes to 0.7 €/kg_{DCW}), 84% (if PHAs content in microorganisms increase to 70%) and 60% (if the energy requirements in ultrasonic extraction are reduced). Likewise, the GW is reduced by 15% by optimizing the ultrasound electricity requirements.

LIST OF PUBLICATIONS

Manuscript I: Rojo, E.M., Molinos-Senante, M., Filipigh, A.A., Lafarga, T., Acién Fernández, F.G., Bolado, S. (2023) "Agricultural products from algal biomass grown in piggery wastewater: A techno-economic analysis". Science of The Total Environment, 887, 164159.

Manuscript II: Rojo, E.M., Rossi, S., Bolado, S., Stampino, P.G., Ficara, E., Dotelli, G. (2024) "Life cycle assessment of biostimulant production from algal biomass grown in piggery wastewater". Science of Total Environment, 907, 168083.

Manuscript III: Rojo, E.M., Piedra, I., González A.M., Vega, M., Bolado S. (2021) "Effect of process parameters on the valorization of components from microalgal and microalgal-bacteria biomass by enzymatic hydrolysis". Bioresource Technology, 335, 125256.

Manuscript IV: Rojo, E.M., Filipigh, A.A., Bolado, S. (2023) "Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass". Process Safety and Environmental Protection, 174, 276-285.

Manuscript V: Rojo, E.M., Hurtado, M., Filipigh, A.A., Ciardi, M., Acién Fernández, F.G., Bolado, S. "Effect of veterinary antibiotics and heavy metals in the composition and valorization of a consortium of microalgae and bacteria". *Submitted for publication in Journal of Environmental Management (JEMA-D23-16222).*

Manuscript VI: Rojo, E.M., Molinos-Senante, María, Irusta, R., Bolado, S. "Ex-ante economic and environmental assessment for fractional valorization of biomass grown on wastewater treatment photobioreactor". *Submitted for publication in Science of Total Environment (S-23-53671).*

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

1.1. Piggery wastewater management

Livestock farming has increased in recent years in Spain, which is the second largest country in pork production in the European Union (Sepúlveda-Muñoz et al., 2023). In 2021, the total number of pig heads in Spain was approximately 335,000,000, which correspond to an increase of ~100% since 1990 according to the "Ministry of Ecological Transition" ("Sistema Español de Inventario de Emisiones," n.d.). All these animals produced more than 70,000 tonnes of pig manure every year, which has become a serious problem for the environment with several negative impacts, including greenhouse gas emissions, eutrophication, acidification, and biodiversity loss (López Fernández et al., 2023; Sepúlveda-Muñoz et al., 2023). Also, this type of residue is characterized by the high presence of organic substances and suspended solids (Kushwaha et al., 2023). Due to all these issues, it is necessary to develop a sustainable management of this residue to minimize its impact in the environment (López Fernández et al., 2023).

1.1.1. Conventional piggery wastewater treatment

The management of this waste has been carried out by different conventional methods that include anaerobic digestion, aerobic/anaerobic/anoxic systems, or direct application as biofertilizer in agricultural crops (Akizuki et al., 2021).

Anaerobic digestion consists of the microbial hydrolysis and bioconversion of organic matter in absence of oxygen (O_2) into biogas composed of 65-75% of methane (CH_4) and 35-45% of carbon dioxide (CO_2) (Deena et al., 2022; Rivera et al., 2022). Currently, this technology is used in treating swine manure as a mature and environmentally friendly process (Zhang et al., 2023) and many studies have confirmed the feasibility of biogas and energy production using livestock wastes as organic matter (Hollas et al., 2023). Methane yield production from swine wastewaters can range from 91 to 300 ml $CH_4/g_{\text{chemical organic demand}}$ with chemical organic demand (COD) removal up to 90% (Cheng et al., 2020). Nevertheless, piggery wastewater usually has a low carbon-nitrogen ratio (compared with other organic substrates) which could limit nutrient recovery and the correct digestion performance (López-Serna et al., 2019). Also, the digested waste may cause eutrophication if it is directly discharged into the environment (Zhang et al., 2021).

On the other hand, aerobic/anaerobic/anoxic treatment focus on the removal of N by decomposition of the ammonia in two steps: oxidation of ammonia into nitrate/nitrite by aerobic bacteria (nitrification) and then, conversion of nitrate/nitrite to N by anoxic bacteria (Kushwaha et al., 2023). This method can achieve a significant removal of COD (>90%), total nitrogen (>90%) and total phosphorus (>80%) from the swine wastewaters. However, the main bottleneck of this technology is the high oxygen necessity for the nitrification step (> 2 mg/L) which entails high aeration costs in the process (Akizuki et al., 2021).

Finally, swine manure can be used as organic fertilizer by direct application in the land. Organic fertilizer can benefit soil quality by applying the N and P present in organic waste although the European Union has a strict regulation on livestock manure use as organic fertilizer (Lessmann et al., 2023) which currently limits their use at the expense of chemical fertilizers. Even so, this is the simplest option for pig manure management, although is associated with health risks and soil contamination, so composting beforehand is recommended (Nagarajan et al., 2019a). In fact, this last process is increasingly used due to the higher demand of food grown with organic fertilizers (López Fernández et al., 2023).

1.1.2. Microalgae for piggery wastewater treatment

Currently, the treatment of piggery wastewater with microalgae has become an innovative process with significant benefits within the circular economy and biorefinery (Chaudry, 2021) in comparison with conventional treatment processes. These photosynthetic microorganisms have the ability to use nutrients such as nitrogen (N) and phosphorus (P) for cell growth by bioconversion (Pradhan et al., 2023) and the ability to fix carbon dioxide (CO_2) from different sources (Sánchez-Zurano et al., 2021a). Nevertheless, microalgae production requires a huge quantity of water and nutrients. Thus, an inexpensive source of these two inputs may be wastewater streams (Pradhan et al., 2023), and specially piggery wastewater which has a high concentration of organic matter, volatile fatty acids, and nitrogen (Plöhn et al., 2021; Sepúlveda-Muñoz et al., 2023). This would allow for a reduction of production costs.

The biochemical process developed during the treatment of wastewater with microalgae is called “photosynthetic oxygenation” (Figure 1), where microalgae and bacteria present in the wastewater work symbiotically. During the process, the O_2

produced by microalgae is taken up by bacteria as an electron acceptor for organic valorization. Then, the CO_2 produced by bacteria during mineralization is taken up by the algae coupled with vitamin B12, N_2, and siderophores required for microalgal functional processes (Khan et al., 2022). This biotechnology is a sustainable and commercially viable platform to treat wastewater and produce valuable biomass in comparison with individual microorganisms, increasing the efficiency of the wastewater treatment process (Zhang et al., 2020a). Finally, the produced biomass can be utilized for various applications, including biochemical production (proteins, carbohydrates and/or lipids), animal feed, biofertilizer, and biofuel (Aditya et al., 2022).

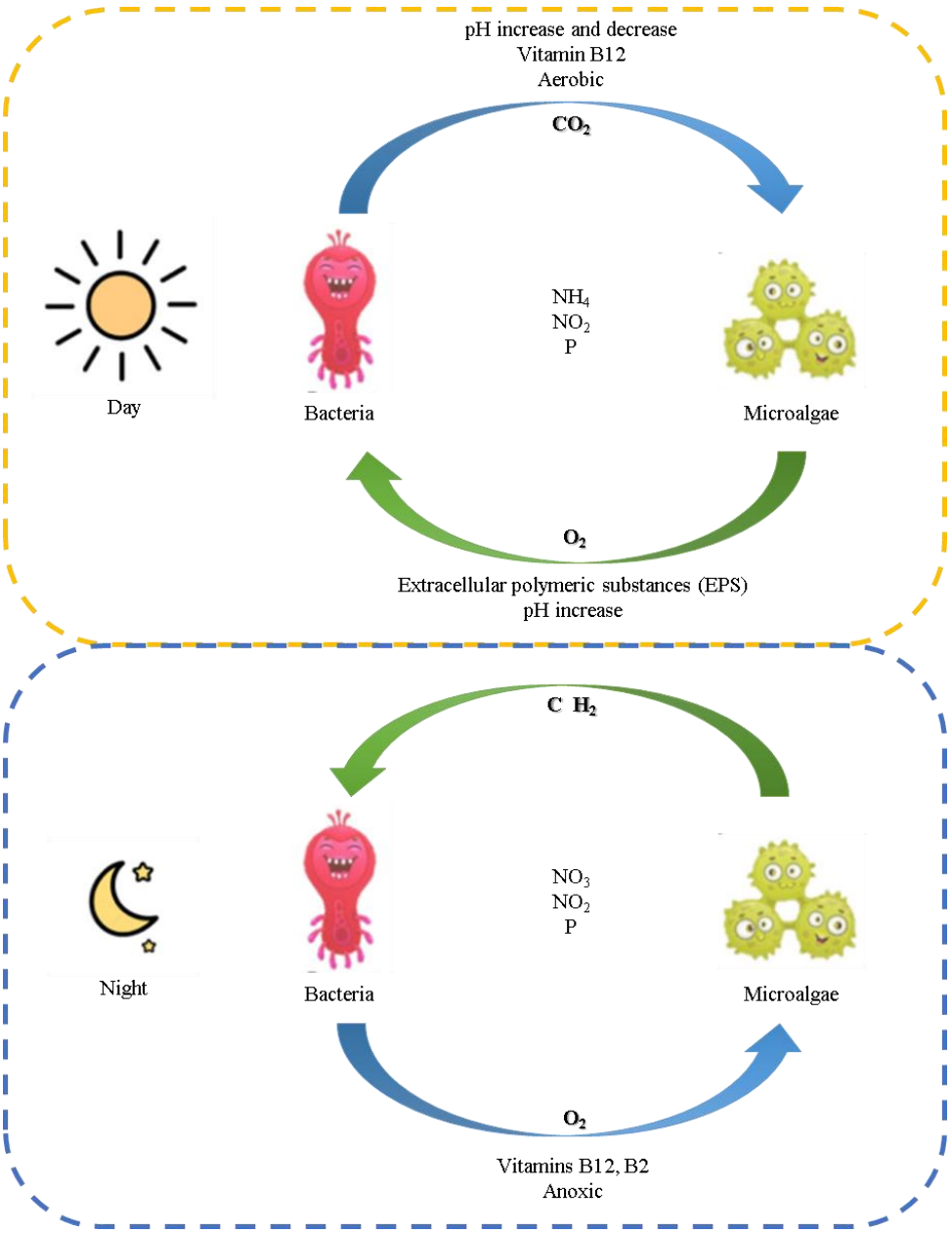

Figure 1. Photosynthetic oxygenation in a microalgae-bacteria consortium based on Aditya et al. (2022).

Likewise, the biomass produced is not only formed by microalgae, but also by bacteria from the treated wastewater that favor the bioremediation process (Figure 1). Normally, the microbial communities found in biomasses cultured in piggery wastewater are formed by heterotrophic bacteria and/or coliforms bacteria such as phylum *Proteobacteria*, *Bacteroides*, and *Firmicutes* (García et al., 2017; Sánchez-Zurano et al., 2021a). The percentage of these bacteria can reach up to 40% of the total biomass (Sánchez-Zurano et al., 2021a) and has also certain influence in the posterior valorization process.

1.2. Microalgae biomass

Microalgae are microorganisms known for their high productivity. They can produce and accumulate significant amounts of biomolecules such as lipids, carbohydrates, antioxidants, pigments, and proteins. From an industrial point of view, microalgae culture has received significant interest due to the high growth rate of microalgae. However, despite microalgae's remarkable capacity to produce bioactive molecules, the extraction and recovery of microalgae components is not straightforward, and extensive research is currently being developed in this field. These drawbacks are related to microalgae cytology, especially to the rigid cell wall of these microorganisms. Microalgae cells have a cell wall and a plasma membrane encapsulating the cytosol which contains a defined nucleus. While the main organelles show a similar chemical composition compared to other organisms, the cell wall's chemistry varies significantly among microalgae species (Alhattab et al., 2019). However, some general trends can be stated: the plasma membrane is made of phospholipids and transmembrane proteins, while the cell wall is formed by cellulose fibers, hemicellulose, β-glucan, and proteins, which confers its harshness. Sometimes a mucilage layer can also be found, composed of alginate and extracellular matrix, acting as a defense layer (Borowitzka, 2018; Tebbani et al., 2014).

The cell contains bioactive compounds which are usually classified into primary and secondary metabolites, depending on their biosynthetic origin, chemical composition, or function. Primary metabolites are produced as a result of cell growth, cell development, and microalgae reproduction and mainly include protein, carbohydrate, lipids, and photosynthetic pigments. Secondary metabolites are uniquely accumulated to relieve cellular injuries under stress condition and consist mainly of carotenoid, phytosterols, and phenolic compounds. Some carotenoids, such as lutein and

fucoxanthin, are components of the light-harvesting complex for photosynthesis and photo-protection and can thus be considered primary metabolites (de Morais et al., 2015). On the other hand, microalgal lipids can be mainly divided into membrane lipids (consisting of polar lipids) and storage lipids (consisting of neutral lipids mainly in the form of triglyceride). Similarly, carbohydrates can be divided into structural carbohydrates and storage carbohydrates (such as starch, glycogen, and glucan) (Ma et al., 2020).

1.2.1. Composition and structure

The microalgae composition depends mainly on the strain and cultivation conditions. The concentration of the three main macronutrients in microalgae can vary widely: the concentration of crude proteins can lay between 17 and 71%, lipids can represent up to 47% (although values around 5-15% are the most common), and the concentration of carbohydrates can be anywhere from 10 to 57% (Grossmann et al., 2020).

Among the common macronutrients found in microalgae, proteins are the most important, reaching up 71% in some species. Water-soluble proteins that can be liberated from algal cells varies between 21-90% depending on species and the extraction technique (Grossmann et al., 2020). It is worth noting that the "crude protein" is usually overestimated, as it includes other non-protein nitrogen compounds, such as nucleic acids (which for some species account for 3-6%), pigments, glucosamides, or inorganic components (Becker, 2007; Bulgariu and Gavrilescu, 2015). Proteins are crucial in microalgae metabolism and are involved in growth and maintenance processes. They also act as chemical messengers, regulators, and provide defense against other microorganisms. Nowadays, microalgae's protein quality is beyond doubt due it is known for its excellent digestibility, and it provides in high amounts the nine essential amino acids that humans do not synthesize (Acquah et al., 2020). This, coupled with their high biomass productivity rates, makes microalgae a potential answer to worldwide protein demands.

Proteins may be found within the plasma membrane and in the cell wall (as transmembrane proteins) or bound to the membrane's lipids (as periphery proteins) (Figure 2). They are also found in the cytoplasm or as part of many organelles such as chloroplast, mitochondria, the endoplasmic reticulum, or inside the cell's nucleus (Safi

et al., 2014b). Transmembrane proteins have a hydrophobic region in contact with the bilayer membrane which is tightly bound (Safi et al., 2014b).

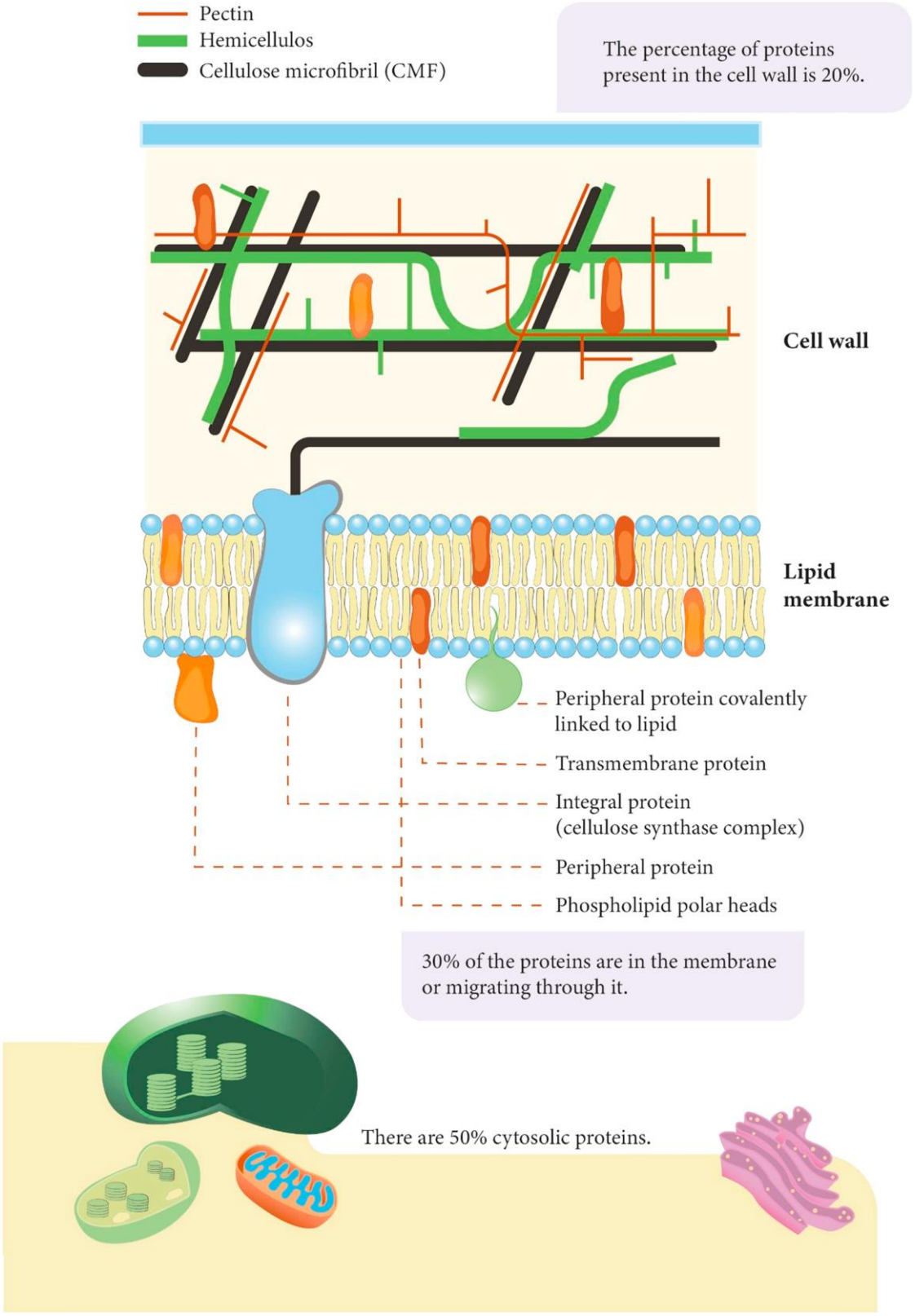

Figure 2. Protein localization on microalgal structure.

The protein concentration of the cell depends on their place in the cell and the microalgae species studied. For instance, *Chlorella vulgaris* accounts for 42-58% of proteins in dry biomass weight, and it is estimated that 20% of its total proteins are found in the cell wall, around 30% actively migrate through the cell, and 50% are located in the cytoplasm (Berliner, 1986). Hence, as roughly 50% of the proteins in microalgae are related to the cell membrane and to increase the recovery rate, it is necessary to disrupt the cell wall's multiples layers to release the components and enhance the subsequent extraction and isolation steps (Phong et al., 2018a).

1.2.2. Influence of toxic elements and emerging contaminants from wastewater

In the case of biomass grown in wastewater, the presence of some toxic elements as heavy metals and emerging pollutants as veterinary drugs in this type of waste must also be considered. Heavy metals, such as zinc or copper, are usually used as feed additives to satisfy the nutritional requirements and prevent nutritional deficiencies (Hejna et al., 2021) while arsenic is found in the well water used on the pig farms in regions where this metalloid is present in the rock matrices of aquifers (Collao et al., 2022). On the other hand, veterinary antibiotics are used for the preservation of animal health (Michelon et al., 2022). In both cases, absorption is not complete by the animals and therefore part of them is excreted in the swine manure (Michelon et al., 2022), from where they are removed by microalgae biomass in a process called phycoremediation.

After a successful phycoremediation, biomass contains some of these contaminants due to the adsorption removal processes, which could affect the composition, structure (Leong and Chang, 2020) and subsequent valorization (Saavedra et al., 2019). Heavy metal has a great influence on the physiological and biochemical processes in algae, such as growth, photosynthesis, cell ultrastructure, protein content and fatty acid composition (Xiao et al., 2023). On the other hand, veterinary antibiotics or their degradation products can inhibit microalgal photosynthetic capacity, cell proliferation, and growth (Yu et al., 2022). To protect against the oxidative stress caused by these pollutants, the biomass changes cell morphology and intracellular ultrastructure, such as cellular size enlarging, cytoplasmic vacuolization, cellular debris. These changes also affect subsequent valorization, as extraction methods depend on a variety of factors including cellular structure and composition.

1.2.3. Extraction of cell components

In literature, it is well documented that microorganism's cell wall prevents the extraction of its components, acting as a protective. Safi et al. (2014a) investigated how the microalgae cell wall may hamper protein extraction. They calculated a proportion factor between the proteins determined by the Lowry method (the hydro-soluble proteins) and the total protein content (estimated with the nitrogen-to-protein conversion factor through elemental analysis) in five different microalgae species: *P. Cruentum, A. Platensis*, *C. Vulgaris, N. Oculate,* and *H. Pluvialis*. The results showed that more hydro-soluble proteins were obtained when the cell wall was more labile, achieving 90% and 78% of protein yield for *P. Cruentum* and *A. Platensis*. For the other three microalgae tested, the amounts diminished, obtaining lower ratios of 52.8%, 52.3%, and 41% from *C. Vulgaris, N. Oculate,* and *H. Pluvialis*. It is well known that *P. Cruentum* does not have a proper cell wall, whereas green microalgae (which comprise the other four) are credited for having a more rigid cell wall.

Hence, the results indicate that the cell wall significantly determines the process extractability output, and it is vital in obtaining good assimilation, bioavailability, and solubilization of proteins. It is crucial that to get the advantage of the whole proteins' potential, they must be smoothly released to preserve their structural identity and functionality (Grossmann et al., 2018). However, proteins are not the only components released after cell wall breakthrough. The protein recovery requires separating proteins from other cell components like lipids, carbohydrates, and less concentrated substances such as pigments or nucleic acids, which are not commonly considered when dealing with microalgae composition. Additionally, an optimal extraction method should be easy to perform, consume small amounts of energy, and provide high disruption yields in short times (Soto-Sierra et al., 2018a). From an industrial point of view, the process should ideally produce as little reagent waste as possible (Phong et al., 2018a)

1.3. Extraction methods

The choice of the extraction treatment depends on the resistance of the raw biomass, the desired product properties, and other economic, technical, and environmental aspects. Mild disruption methods are usually used to avoid damage to the proteins and to preserve their techno functional properties (Callejo-López et al., 2020).

The wide range of treatment options is often classified as physical, chemical, and biological methods (Figure 3).

Selecting one method or a combination of methods to recover the protein fraction will be conditioned by the microalgae cell wall and the proteins application. For example, if proteins must maintain their structural integrity and functionality, physical methods are recommended as they are milder and usually avoid damage to the proteins and preserve their techno-functional properties (Callejo-López et al., 2020). In this case, special attention must be paid to the possible overheating and, as consequence, the protein degradation. The main disadvantage of these physical methods is their low extraction yields. For example, comparing ultrasonication and chemical treatments for *H. Pluvialis*, the protein extraction yields obtained were 13.5% and 31.1%, respectively (Safi et al., 2014a).

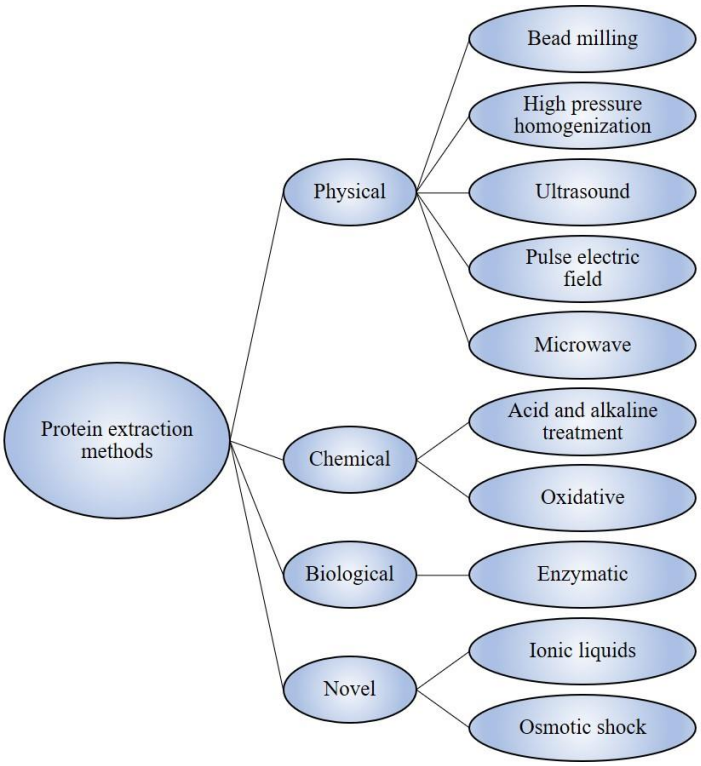

Figure 3. Classification of the most studied methods for protein extraction.

On the contrary, if looking for a higher protein output, the use of chemical agents, and combined or assisted physicochemical techniques are preferred to break covalent bonds of the cell wall more efficiently. However, these methods can degrade proteins through denaturation, cross-linking, racemization, or hydrolysis reactions (Amorim et al., 2021). Therefore, a compromise solution must be found between a good recovery yield, energy consumption, and product degradation (Alhattab et al., 2019).

1.3.1. Physical methods

a) Bead milling

Bead milling is one method for cell disruption of microalgae to extract different internal compounds based on direct mechanical damage on the cell wall. It uses beads inside the milling chamber and the cell wall disruption is caused by several processes: collision of cells with these beads due to differences in velocities, shear stress due to the acceleration of beads towards the milling chamber wall, and centrifugal acceleration of the mill wall (Nitsos et al., 2020). Advantages such as high cell wall disruption efficacy, high biomass loading, temperature control, commercially available equipment, quickly and easily scale-up, and low labor intensity makes bead milling an efficient technique for protein extraction (Timira et al., 2021). However, this technology has also drawbacks such as high energy demand at large scale (which makes this method unsustainable for microalgae biorefineries) and the nonselective extraction of biomolecules from the microalgae biomass (Soto-Sierra et al., 2018a; Timira et al., 2021)

The most important parameters are milling chamber geometry, microalgal biomass concentration, agitator speed, suspension flow rate, bead filling ratio, bead type, and bead diameter (Postma et al., 2017). These parameters influence the efficiency of cell disintegration and, therefore, of protein extraction. Several articles have investigated this method for the extraction and recovery of proteins from microalgae and have confirmed that it is one of the most effective techniques. Alavijeh et al. (2020) obtained a protein recovery yield of 40% from *Chlorella vulgaris* after only 10 min in a horizontal 75 mL bead mill chamber with a 65% filling percentage by 0.4 mm Y_2O_3 stabilized ZrO_2 beads, a constant agitation speed of 2039 rpm. and a biomass concentration of 25 g/L. Also, subunits of RuBisCo, a protein enzyme with sizes between 14 and 56 kDa, were recovered after the process.

b) High pressure homogenization

High pressure homogenization (HPH) is a mechanical process, during which a microalgae biomass suspension is forced by high pressure (50–300 MPa) through a micrometric disruption chamber, where the velocity increases rapidly (Carullo et al., 2020). Therefore, cell wall rupture occurs due pressure drop, shear stress, cavitation, turbulence, and impingement of the cells to the surface of the valve at high velocities

(Nitsos et al., 2020). The most important parameters that affect the process are the operation pressure, number of cycles, and fluid dynamics such as flow rate (Timira et al., 2021). HPH is one of the most effective rupture techniques for compound extraction from microalgae, including proteins. It can be scaled, is easily applicable to highly concentrated algal pastes, is relatively energy-efficient, and the cell disruption rates are high compared to pulse electric field, acid, or alkaline treatment (Timira et al., 2021).

Using HPH has several disadvantages and drawbacks (low dry cell concentrations, difficulties in breaking rigid cell walls, and nonselective intracellular compound release) (Timira et al., 2021). This method always requires an efficient heat depletion at the homogenization valve because of the high temperature increase that occurs (Nitsos et al., 2020), which can degrade the extracted proteins and can cause reversible or irreversible alteration of the tertiary and quaternary structure of proteins (Carullo et al., 2020). Also, the shear stress and high pressure used can damage protein properties.

Carullo et al. (2018) extracted proteins from *Chlorella vulgaris* with a yield of 54.1% after 5 HPH passes at 150 MPa and 155 mL/min of flow rate, and the protein release had already peaked at a pressure of 100 MPa, with a yield of 50%, indicating that partial cell breakage is enough for the sufficient extraction. Elain et al. (2020) achieved a protein yield solubilization of 62.7% using *Arthrospira platensis* and a two-stage homogenizer with an inlet pressure of 500 bar and 9 L/h of flow rate, solid to liquid ratio of 1:6 w/v, and 7 passes.

c) Ultrasonication

Ultrasonication is considered a green extraction technique which presents several advantages in terms of shortening the extraction time, decreasing solvent volumes, and increasing the yield of targeted compounds (like proteins) in comparison with conventional methods (Vernès et al., 2019b). In ultrasound assisted extraction (UAE), ultrasound waves of 20-100 MHz are used to create localized high-pressure bubbles in the liquid that collapse and generate shock waves which causes high shear forces and thus lead to cell wall disruption (Shahid et al., 2020). The main factors affecting the process are the solvent's physical properties such as viscosity, saturation, vapor pressure, surface tension (Vernès et al., 2019b), and process temperature (which can be

significantly increased if not controlled, affecting the quality and properties of the extracted proteins).

The most important parameters are ultrasound power and frequency, process time, microalgal biomass concentration, and type of solvent. For protein extraction, the most used frequencies are between 20 and 40 kHz (Vernès et al., 2019a), with treatment times ranging from 10 min to 2 h. For example, Hildebrand et al. (2020) achieved a maximum protein recovery of 76.6% from a *Chlorella vulgaris* suspension (0.2 g/mL) using an ultrasonic probe at maximum power (1000 W) for 10 min and NaOH 0.4 M as solvent. Using water or HCl 0.4M as solvents, the yield was only 35%. Vernès et al. (2019a) obtained a protein recovery yield of 26.7% after 20 min of process from 1:20 (g/g) of *Arthrospira platensis* suspension in the phosphate buffer using an ultrasonic device at low frequency (20 kHz). The value increased by 6% with the application of 2 bar pressure.

Due to the highly resistant cell wall of most microalgal species, ultrasonication alone is not very effective for the complete extraction of proteins and must be accompanied by other methods, like enzymatic hydrolysis or bead milling (Nitsos et al., 2020; Soto-Sierra et al., 2018a). The combination of various treatments can significantly improve the extraction yield, as shown by Hildebrand et al. (2020) who obtained a protein recovery of ~45% when a lysozyme treatment of 1 h was combined with 10 minutes of ultrasound pretreatment at 1000 W of power and water as solvent.

d) Pulsed electric field

Pulsed electric field (PEF) is a non-thermal method that disrupts the lipid bilayer of cell membranes allowing molecules of certain sizes, such as small proteins, to enter and/or diffuse out of the cells (Soto-Sierra et al., 2018a). PEF involves applying an external high electric current to increase the transmembrane voltage to perforate and permeabilize the microalgal cell wall (Matos, 2019a).

The most important operational parameters are the electric field strength, wave shape, number and duration of pulses, temperature, and the product and media characteristics (Rocha et al., 2018). Gateau et al. (2021) extracted 10.2 µg/mL of proteins from *H. pluvialis* using an electric filed strength of 1 kV/cm and 5 pulses from a microalgae suspension of 10^5 cells/mL at 10ºC. Buchmann et al. (2019), using a field strength of 20 kV/cm, achieved an extracted protein concentration of 0.5 g/L from a

microalgae concentration of 6 g/L, a lower value than the concentration reached after HPH treatment (2.75 g/L) of the same microalgae.

As can be concluded from the results described above, PEF could be used as a supplementary treatment and it has been successfully applied for protein extraction at low energy intensities (Timira et al., 2021). However, it is not an efficient disruption method for complete protein extraction. When complete solubilization and extraction of microalgal proteins is required, energy-intensive cell disruption methods, or a combination of more than one method, is recommended (Soto-Sierra et al., 2018a).

e) Microwave

In microwave assisted extraction (MAE), the process acceleration and the increase of extraction yields are the result of a synergistic combination of two transport phenomena: heat and mass gradients (Vernès et al., 2019b). Microwaves offer fast heating in comparison to conventional heating and selective energy dissipation and can cuts down working times (Shahid et al., 2020). MAE consists of applying a microwave irradiance at a frequency near 2.45 GHz (Kapoore et al., 2018), causing dielectric heating by absorption of the energy in water and other polar compounds (Timira et al., 2021). It induces the vibration of water and polar molecules within wet microalgae biomass, resulting in temperature increases in the intracellular liquids which causes the solvents to evaporate and exert pressure on the cell walls, leading to disruption (Costa et al., 2020; Kapoore et al., 2018). MAE consumes less solvents, presents higher extraction yields and enhanced efficiency, is nontoxic, can be used for larger volumes with high uniformity, selectivity, and low energy consumption, uses short reaction time, and has low operation costs (Costa et al., 2020; Ventura et al., 2017).

The most important operational parameters are microwave irradiation time, duty cycle, microwave power, and solvents (Chew et al., 2019). Passos et al. (2015) obtained a protein concentration of 193 mg/L from a mixed culture of microalgae (*Stigeoclonium* sp., *Monoraphidium* sp., *Nitzschia* sp. *and Navicula* sp.) when they applied a microwave power of 900 W during 3 min with water as solvent (initial biomass concentration of 3% w/w with 58% of proteins and 22% of carbohydrates). It is commonly used in combination with other treatments, increasing their efficacy. For example, Chew et al. (2019) recovered 63.2% of proteins from microalgae *Chlorella vulgaris* using a microwave time of 120 s, duty cycle of 80%, biomass concentration of

0.5% w/w and 100W of power combined with three phase partitioning (TPP) using ammonium sulphate at a concentration of 30% and a ratio of slurry to t-butanol 1:1.

1.3.2. Chemical methods

a) Acid and alkaline treatment

Acid and alkaline treatments involve exposing the microalgal biomass to an acid or alkaline aqueous medium. In some cases, this pretreatment is combined with elevated temperature (120-160ºC), in which case it can also be considered a hydrothermal pretreatment variation with the addition of an acid and basic solvent that acts as catalyst (Nitsos et al., 2020). The most used acid solvents are HCl and H_2SO_4 with a concentration between 1 and 5%. These solvents cause the biomass to swell and degrade the cell wall polymers (Salakkam et al., 2021). In contrast, alkaline treatment consists of treating the algal biomass with an alkaline solvent, usually an aqueous NaOH solution. This method is used to disintegrate and disrupt the microalgal cell wall and solubilize organic molecules, particularly protein (Salakkam et al., 2021). It has been demonstrated that acid and alkaline treatments disrupt the cell wall of microalgae and facilitate protein extraction, although when combined with heat, these methods can degrade and modify protein properties due to denaturation and racemization (Callejo-López et al., 2020). They can also lead to the formation of amino acid complexes through Maillard reactions and limiting the availability of amino acids in the extracts (Timira et al., 2021).

Advantages of these chemical methods include high efficiency, low energy input, and easy scalability. Nevertheless, they are not considered to be mild and can have serious effects on protein, as described above. Therefore, due to the aggressive nature of these methods, careful process conditions are required to avoid the degradation of the extracted protein (Nitsos et al., 2020). In addition, they show low selectivity, causing the release of multiple components which results in difficult posterior separation. These are the principal drawbacks that need to be investigated before applying them.

Callejo-López et al. (2019) designed an alkaline process for the extraction of proteins from fresh microalgae biomass (consortium formed with *Chlorella vulgaris, Nannochloropsis gaditana, and Scenedesmus obliquus*) at 60 mg/mL and 100 mg/mL. They achieved a final protein recovery yield of 87.5%, after an alkaline process of 2 hours at 50ºC and pH 13.4 (50mM Na_2HPO_4 and NaOH titration). For the acid method,

Martin Juárez et al. (2021) obtained protein solubilizations of 47.3% and 75.5% from fresh algal-bacterial biomass composed mainly of *Scenedesmaceae* grown on pig manure after acid treatments with 0.5 M HCl and 2 M HCl, respectively, during 1 hour at 121ºC.

b) Oxidative treatment

This method consists of exposing the microalgae to oxidative agents, such as ozone or hydrogen peroxide (Nitsos et al., 2020), which can be very aggressive to the biological biomass. Ozone is a strong oxidant that can destroy several types of microorganisms and attack the cell wall (especially the double bonds in membranes), resulting in damage to the cell structure (Keris-Sen and Gurol, 2017). It has proven to be attractive alternative over other extraction methods due to its low production of inhibitory compounds, low chemical consumption, mild operational conditions, and generation of easily degradable byproducts (González-Balderas et al., 2020). Among the disadvantages are the highly reactive, flammable, corrosive, and toxic characteristics of ozone, the exothermic process, the necessity of special construction materials, and the high costs generation (Travaini et al., 2016b). One recent investigation achieved a protein release of 58% from *Desmodesmus* sp. at an ozone concentration of 45mg/L, a contact time of 35 min, and alkaline conditions (pH 11) (González-Balderas et al., 2020). On the other hand, hydrogen peroxide (H_2O_2) is an alternative oxidizing agent for the pretreatment of microalgae with a similar mechanism to ozonation. Duan et al. (2017) obtained a protein extract content of around 25 μg/cm^3 from *Chlorella pyrenoidosa* at a cell concentration of 12.5 × 10^7 cells/mL by applying a combined treatment of ultrasounds (20 min and 35 Hz) and H_2O_2 (0.1 mM).

1.3.3. Biological methods

a) Enzymatic hydrolysis

Enzymatic hydrolysis is a green alternative to traditional physical and chemical methods which permits a selective extraction of all the biomass components, including proteins (Timira et al., 2021). This method is based on the use of different types of enzymes under mild conditions (proteases, cellulases, and lipases) to degrade the complex cell wall which is composed mainly of carbohydrates, proteins, and organic polymers (Nitsos et al., 2020).

Since this method can be operated under mild and gentle operating conditions, serious damage to the intracellular compounds can be avoided while operating at low temperatures with low energy demand (Nitsos et al., 2020; Phong et al., 2018b). There are many variables which could affect the enzymatic activity. These variables include the characteristics and concentrations of the enzymes, the intracellular composition, the cell wall composition, the type of microalgae, and temperature, although the most important variable is the type of enzyme used (Phong et al., 2018b). Enzymatic methods which involve the use of proteases have been proven to be highly efficient in amino acid production from microalgae (Callejo-López et al., 2020). The potential limitations of this method include the cost of commercial enzymes, the lack of knowledge about optimal or compatible enzyme formulations for cell disruption, and the requirement for holding tanks to accommodate long incubation periods (Dixon and Wilken, 2018a; Salakkam et al., 2021).

Various studies have studied the efficiency of this method. Corrêa et al. (2021) obtained a protein solubilization yield of 11.6% from fresh microalgae biomass mainly formed by Scenedesmaceae grown on pig manure. In this case, enzymatic hydrolysis was performed with Celluclast 1.5L and Novozyme 188 and an enzymatic hydrolysis time of 12 h. Callejo-López et al. (2019) achieved an optimum protein recovery yield of 40-50% from a consortium biomass formed by *Nannochloropsis* sp. by applying enzymatic hydrolysis with Alcalase 2.5L, at temperature of 60 ºC, a hydrolysis time of 2 hours and after an alkaline pretreatment of 2 hours and pH of 13.4. However, the application of enzymatic hydrolysis alone only achieved a protein solubilization between 30.5% and 46%.

1.3.4. Novel methods

a) Ionic liquids

Ionic liquids (IL) are organic salts in the liquid state with low melting points below 100ºC (Nitsos et al., 2020). They consist of a large asymmetric organic cation and an organic or inorganic anion. The IL's ion can modify the cell wall because of their high hydrogen bond accepting ability, enhancing the protein extraction (Timira et al., 2021). Ionic liquids present some attractive characteristics such as a low-melting point, extremely low volatility under atmospheric conditions, the capability of dissolving a wide range of polar to non-polar compounds, low flammability, and high thermal and

chemical stability (Phong et al., 2018b). Despite these advantages, few research has focused on this method, due to its complexity, the fact that some ILs are not environmentally friendly, and the laborious purification process (Timira et al., 2021). Suarez Garcia et al. (2023) extracted protein from the macroalgae *Ulva lactuca* using ethyl methyl imidazolium dibutyl phosphate [Emim][DBP] as IL and achieved a recovery of ~75% (biomass concentration of 1 wt%, room temperature and IL concentration of 40%).

1.4. Valuable products from algal biomass grown in wastewater

Due to the composition of microalgae and richness of different biomolecules, this type of biomass can be a source valuable biomolecule such as proteins or carbohydrates and/or be used to obtain added value products, including bioplastics, biofuels from carbohydrate fraction, and agricultural fertilizers, pesticides, animal feed, peptides for industrial applications (foaming and emulsifying) or pigments from protein fraction (Malik et al., 2023; Sivaramakrishnan et al., 2022). However, since the culture media used for biomass production is wastewater, proteins obtained through these processes cannot be used for human consumption (Lorenzo-Hernando et al., 2019). Thus, this biomass can be considered a promising feedstock in a biorefinery approach within the circular bioeconomy to obtain all these value-added products from wastewater (Moreira et al., 2023). According to Chew et al. (2017), biorefinery is a "*process to obtain biofuels, energy and high-added value products through biomass transformation and process equipment*". The main objective of this technology is to obtain multiple products from only one biomass (in this case, microalgal biomass grown on wastewater). This emerging process can allow to reduce greenhouse gas emissions and minimize environmental wastes but has also some bottlenecks which make currently unsustainable (Severo et al., 2021). The principal challenge is the extraction and separation of the different fractions in the downstream step, which increases the cost of the biorefinery process (Gifuni et al., 2019a). So, the integration of multi-bioproduct production from only one biomass must be explored to reduce the operating costs of microalgae biorefinery by increasing sales revenue (Sivaramakrishnan et al., 2022).

1.4.1. Proteins

The major component of algal biomass is protein (17 - 71%), which makes microalgae a great renewable source of this type of biomolecule. There are many uses

for microalgal biomass protein, including animal nutrition in livestock or aquaculture (Behera et al., 2022), industrial applications with functional properties (gelation, foaming and emulsifying properties) (Kumar et al., 2022), biostimulants, or pigments source like phycobiliproteins (Chini Zittelli et al., 2023). Microalgae proteins have been reported to possess bioactive properties (Table 1) linked to an inherent sequence of aminoacid forming small peptides with chain lengths ranging from 2 to 20 AA (Kumar et al., 2022). These bioactive properties were also found recently from peptides derived from hydrolysis that transform large proteins into smaller molecule (Srimongkol et al., 2022), so the best way to exploit these properties is to hydrolyze the biomass with the different techniques proposed in section 1.3 below. However, due to the presence of bacteria from wastewater, proteins obtained through these processes cannot be used for human consumption.

Macrocompound	Microalgae	Active compound	Properties
Protein	*Chlorella sorokiniana*	9.9% protein solution	Maximum elastic modulus 238.4 Pa[B]
	Chlorella pyrenoidosa	3% protein solution	Foaming capacity and stability up to 95%[B]
	Chlorella vulgaris	Short peptide <1.3 kDa	Antioxidant properties[A]
	Schizochytrium limaciumn	Short peptide 5-10 kDa	Antioxidant properties[A]
Carbohydrate	*Chlamydomonas reinhardtii*	Ara, Rha, Rib, Xyl, Gal, Glc	Antioxidant properties[A]
	Arthrospira platensis	Polysaccharides extracts	Tomato plants significantly improved the nodes number, shoot dry weight, and shoot length[C]
	Spirulina platensis	Sulfated polysaccharide	In vitro antioxidant activity, antibacterial activity[C]

Table 1. Bioactive properties of microalgae macrocompounds. [A](Srimongkol et al., 2022), [B](Kumar et al., 2022), [C](Moreira et al., 2022).

Regarding this bio-compound, it must be taken into account that they are very sensitive to harsh conditions (low pH or high temperatures), so protein extraction would

be the first step to be considered in a biorefinery approach to preserve their properties as much as possible (Lorenzo-Hernando et al., 2019).

1.4.1.1. Biostimulants and biofertilizers

Biostimulants are compounds which produce an increase in crop yield of plants via metabolism stimulation, nutritional optimization, and resistance to abiotic processes (González-Pérez et al., 2022a). Many studies have proven the efficiency of the use of microalgal biomass extracts to improve germination index and absorption of nutrients of the plants (Moreira et al., 2023). Also, it improves soil characteristics (stability, enzymatic and microbiological activity) (Braun and Colla, 2022). The phytohormones, polysaccharides and amino acids are responsible for growth stimulation, while other secondary metabolites with bioactive properties are responsible of soil improvement (Ferreira et al., 2023; Moreira et al., 2022). Several authors have proven the efficacy of these bioproducts obtaining from microalgae biomass grown on piggery wastewater in improving germination index of cucumber by 75-138% (Ferreira et al., 2021) and wheat by 45% (Ferreira et al., 2022) (Table 2).

	(Ferreira et al., 2021)	(Navarro-López et al., 2020a)	(Ferreira et al., 2022)
Microalgae specie	*Chlorella vulgaris*	*Scenedesmus* sp.	*Tetradesmus obliquss*
Cultivation conditions	1:20 PWW Bubble column photobioreactor Temperature 23-25ºC 53 µE/m^2/s	1:10 PWW Open thin-layer cascade photobioreactor Dilution rate 0.3 d^{-1}	1:20 PWW
Treatment	Biomass concentration by settling	High pressure homogenization (200 – 800 bar) and enzymatic hydrolysis with Alcalase 2.5L and Flavourzyme 1000L	High pressure homogenization (100 – 600 bar)
Dosage	0.5 g/L of microalgae culture	0.1 – 1 g/l of microalgae extracts	0.74 – 0.84 g/L of microalgae extracts

Results	Increased germination index of cucumber seeds by 75-138%	10% increase on germination index after HPH at 200 bar	45% increase of wheat germination after HPH at 100 bar

Table 2. Biostimulant production from microalgae biomass grown in piggery wastewater.

1.4.2. Carbohydrates

These biomolecules (with a content in microalgae biomass between 10 and 57%) are the main product from the photosynthetic pathway in microalgae growth (de Carvalho Silvello et al., 2022). Commonly they are mainly formed by glucose, xylose and/or galactose and found as structural polymers in the cell wall (Moreira et al., 2022).

Currently, these biomolecules are usually used to produce biofuels such as bioethanol and/or biohydrogen (Chew et al., 2017; Li et al., 2023). For bioethanol production, the biomass must be hydrolyzed to transform complex sugars into simple monosaccharides before the fermentation of carbon by specific microorganisms, while biohydrogen is produced by different fermentation processes (dark fermentation and/or photo fermentation) or by biological photolysis (Srimongkol et al., 2022). Because these molecules are confined within a rigid cell wall, pretreatment is also necessary prior to the fermentation of biological transformation (de Carvalho Silvello et al., 2022) to improve biofuels production. This pretreatment includes physical, chemical, or biological methods (section 1.3). However, biofuel production from microalgae is still unfeasible in comparison to fuel production from fossil sources.

Thanks to its rheological and biological properties in recent years, it has gained use in animal feed (with antioxidant, antibacterial and antiviral properties) (Moreira et al., 2022) (Table 2). Also, microalgal polysaccharides can be used to produce commercially valuable components such as hydrophilic greases and thickening agents, such as agar (Chandrasekhar et al., 2022).

1.4.2.1. Bioplastics

Conventional plastics are formed by petrochemical polymers, including polyethylene (PE), polypropylene (PP) and polyvinyl chloride (PVC), which are difficult to decompose under ambient conditions as they exhibit a great resistance against microbial degradation. This results in a high environmental impact and in recent

years, research has been carried out into the production of bio-based biodegradable plastics such as polyhydroxyalkanoates (PHA) to substitute petroleum-based plastics.

Polyhydroxyalkanoates (PHA) are produced through microbial fermentation wherein carbon sources are metabolized into PHA and aggregate intracellularly in granules (Tan et al., 2022). Currently, a promising method for producing bioplastics involves the use of microalgae which are capable of photoautotrophically accumulating PHB at concentrations ranging from 0.04% to 80% of their dry mass (Mastropetros et al., 2022). Nevertheless, microalgal carbohydrates are the most used carbon source for PHA-synthesizing bacteria (Tan et al., 2022), but these biomolecules must be extracted within cell wall by several extraction methods explained in section 1.3. Some advantages of bioplastics including high biodegradability, low carbon footprint, energy efficiency and reduction in plastic litter which has increased interest in these high-added products (Arora et al., 2023).

1.5. Environmental and economic sustainability of microalgae biorefinery

Over years, the investigation of microalgal biorefinery has focused on upstream and downstream processes at small scale and despite all the advances achieved, the real implementation at industrial scale of algal biorefinery technologies for bioproducts obtention is still very limited (Behera et al., 2022). So, research should focus more on techno-economic analysis (TEA), life-cycle analysis (LCA) and market analysis (Parsons et al., 2020) to analyze the real feasibility of the microalgal biorefinery and the investigation efforts necessary to achieve industrial usableness in a cost-effective manner (Chandrasekhar et al., 2022).

A techno-economic analysis (TEA) consists of determining the economic performance of the designed microalgae biorefinery process. It must include total capital cost, total production or manufacturing cost and an economic model framed using the capital expenditure estimation and the total revenue obtained from the selling products (Saravanan et al., 2023). With all these tools, it is possible to estimate the economic feasibility of the process. Many publications have performed a TEA to calculate process production costs and feasibility of different biorefinery products and specifically, for biofuel production (Bhatt et al., 2022; Khan et al., 2023). The large variation in results between publications is noteworthy, mainly due to the different processes designed and studied (type of photobioreactor used, the species of microalgae,

the downstream process, operating conditions, etc…). Also, many researchers have identified the bottlenecks with the commercialization and implementation of single product biorefinery from microalgae, so the best way to overcome this issue is a multi-product algal-based biorefinery (Thomassen et al., 2016). However, few studies have performed a TEA for multi-product biorefinery (Lopes et al., 2023; Slegers et al., 2020; Tejada Carbajal et al., 2020), which confirmed the improvement of the economic viability with the production of several products.

On the other hand, life cycle analysis (LCA) is also an important tool for analyzing the ecological effects and impacts related to various bioproducts or processes to quantify the environmental impact and energy requirement (Saravanan et al., 2023). In the specific case of microalgae biorefinery, most studies predict the impact to be uncertain (i.e., both positive and negative) and the generalization of output cannot be done even though the processes have similarity (Chanana et al., 2023; Saravanan et al., 2023). As with TEA, most LCA are focused on the impact evaluation of biofuel production from microalgae biomass (Ubando et al., 2022a) with also a great variability in the results of global warmings potential and functional units. This makes it difficult to properly compare results and thus, it necessary to study each specific process.

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CHAPTER 2: AIM AND SCOPE OF THE TESIS

2. AIM AND SCOPE OF THE THESIS

2.1. Justification of the thesis

Several treatment processes have been investigated in the last years to recover nutrients from piggery wastewater and to reduce its environmental impact. This type of waste is produced in huge quantities in the livestock sector (especially in the swine sector) and has a high content of organic matter, nitrogen and phosphorous, which makes necessary a proper management. The treatment with consortium of microalgae and bacteria has emerged as a green process with viable technical and economic results along with environmental benefits. Reduction of pollutants and pathogens, recovery of nutrients in the form of valuable biomass, energy savings and fixation of CO_2 are some of the advantages of this green technology for piggery wastewater treatment.

The produced microalgal biomass can be used as raw material to produce different valuable bioproducts in a biorefinery process, playing an important role within the bioeconomy framework. It is mainly composed of proteins, carbohydrates, and other bioactive compounds. The microalgae grown on wastewater contain low percentage of lipids, which makes the production of oil-based biofuels unsustainable.

As first and simple alternative, the use of the whole biomass for other applications such as animal feed or agricultural products (biofertilizers and/or biopesticides) would be a good option. Several studies have proved the efficiency of microalgae as biostimulants and its biopesticides activity, but the high cost of producing pure microalgae using synthetic culture media results in non-competitive agricultural products. Therefore, the use of nitrogen rich wastewater as culture media could reduce the production costs while combining wastewater remediation with production of biostimulants and biopesticides. Thus, it is necessary to study the economic and environmental sustainability of these treatment and valorization processes.

On the other hand, the application of a biorefinery concept to carry out a fractional valorization of different microalgal biomass components instead of the whole biomass could provide greater benefits but would require a more complex process. The recovery of components from microalgal biomass requires as first step the breakthrough of the cell wall. The high cell wall resistance of this type of microorganism and the lability of the target components makes the extraction step of these internal compounds a challenge which requires the development of an efficient and environmentally friendly

process. Different extraction methods (chemical, physical and/or biological) and operational methods can be used. Extreme operating conditions (temperature, pressure, pH…) in chemical methods, the most used at present, allows high extraction yields but sometimes with high degradation of the solubilized components. The use of mild operating conditions in biological or physical methods usually results in low extraction yields of high-quality bioproducts and low environmental impact. The selection of the extraction method and operation conditions will depend on the composition and techno-functional properties of the final desired product. Additionally, the presence of pollutants as veterinary antibiotics and toxic components must be monitored through the whole process, to guarantee the safety of the final products.

A possible biorefinery process for a fractional valorization of all the microalgal biomass components is to recover the proteins as peptides and to use the remaining components as culture media for microorganism able to accumulate polyhydroxyalkanoates (PHAs). Proteins are the major and most valuable component of microalgal biomass grown on piggery wastewater and it should be the first recovered component. The recovery of high molecular weight of peptides provides an exhaust biomass rich in carbohydrates and still containing some proteins, useful as culture media. By applying technoeconomic and life cycle assessments, the feasibility of this biorefinery option can be determined and thus, its real viability of the microalgal biorefinery identifying the weaknesses to improve and to address the future research.

2.2. Main objectives

The aim of this thesis is to address the above considerations which the main objective of evaluating and studying different processes for the valorization of microalgal biomass grown on piggery wastewater, focusing on the component extraction alternatives, and considering the contamination by metals and emerging pollutants. More explicit, the following specific objectives are pursued:

- **Objective 1**: evaluate the economic and environmental viability of the use of microalgae for piggery wastewater treatment and the valorization of biomass for agricultural uses, comparing different process alternatives.
- **Objective 2**: evaluate the effect extraction methods and conditions on the solubilization and recovery of the different algal biomass components (proteins and carbohydrates) and its effect on the characteristics of the recovered products.

- **Objective 3**: evaluate the impact and influence of contamination by heavy metals and veterinary antibiotics on the algal biomass composition and extraction processes.

- **Objective 4**: analyze the technical and economic feasibility of a selected fractional valorization process by techno-economic assessment (TEA) and life cycle analysis (LCA), identifying the major hotspots.

2.3. Development of the thesis

In order to achieve these objectives, several assays, experiments and assessments were carried out during the thesis with specific purposes. Initially, we assessed the techno-economic and environmental feasibility of the production of agricultural products (biostimulants and biopesticides) from microalgal biomass grown on a thin-layer photobioreactor treating piggery wastewater (*Chapters 3 and 4*) with the aim of studying the:

- Costs of producing biostimulant as a single product along with the costs of co-production of biopesticide and biostimulants, and comparison with commercial fertilizers.

- Comparison between two different types of harvesting methods (centrifugation and membrane system) and its influence on the viability of the process.

- Parameters that most affect the proposed process in economic terms (raw material and energy prices) by a sensitivity analysis.

- The environmental impacts through a comprehensive life cycle assessment of biostimulant production, comparing the use of CO_2 captured from flue gas with two different technologies (chemical absorption and membrane separation).

Considering that the cell wall breakthrough is a critical step of the fractional valorization processes, we investigated the effect of operational conditions of a biological extraction method (enzymatic hydrolysis) to produce peptides and monosaccharides (*Chapter 5*) with the aim of studying the:

- Effect of microorganisms on the extraction of macromolecular compounds (proteins and carbohydrates) from two different biomasses: pure microalgae

grown on synthetic media and a bacterial-microalgal consortium grown on piggery wastewater.

- Effect of the type of enzyme and the hydrolysis time on the solubilization of the principal microalgal compounds (proteins and carbohydrates) by enzymatic hydrolysis with proteases and cellulases.
- Effect of the type of enzyme and the hydrolysis time on the recovery of peptides, glucose, and xylose by enzymatic hydrolysis with proteases and cellulases.

Besides biological methods, chemical and physical methods are also used for the extraction of macrocompounds with varying results. Chemical treatments provide high solubilization yields but with high degradation while biological and physical methods provide low/moderate extraction yields of good quality products. The combination of different types of methods could result in more effective processes with high solubilization yields and recovery of good quality products. So, a comparison of novel and conventional extraction technologies for the valorization of microalgae biomass grown on piggery wastewater (*Chapter 6*) was performed with the aim of studying the:

- Comparison novel methods as ultrasound and microwave-assisted enzymatic extraction using proteases with conventional chemical methods (alkaline and acid hydrolysis) in terms of solubilization and recovery yields of components and characteristics of products.
- Effect of temperature of the chemical methods (120ºC, 60ºC, and 40ºC) on the solubilization and recovery yields of components and characteristics of products.
- Effect of using an enzyme cocktail of proteases and cellulases in enzymatic hydrolysis, ultrasound, and microwave-assisted enzymatic extraction methods in terms of solubilization and recovery yields of components and characteristics of products.

The use of piggery wastewater for microalgae cultivation can result in a produced biomass with contaminants that were present in the wastewater. These contaminants include emerging pollutants (veterinary antibiotics) and metals with a possible effect on the growth and the structure of the microorganism. So, the influence of these contaminants on the valorization of a microalgal-bacterial consortium grown on piggery wastewater (*Chapter 7*) was investigated with the aim of studying the:

- Effect of three veterinary antibiotics (sulfadiazine, tetracycline, and ciprofloxacin) on the algal biomass composition.
- Effect of three metals (copper, zinc, and arsenic) on the algal biomass composition.
- Effect of three veterinary antibiotics on the solubilization and recovery yields of proteins and carbohydrates and characteristics of products by chemical, enzymatic and ultrasound-assisted enzymatic hydrolysis.
- Effect of three metals on the solubilization and recovery yields of proteins and carbohydrates and characteristics of products by chemical, enzymatic and ultrasound-assisted enzymatic hydrolysis.

Finally, based on the previous results, a fractional valorization process for microalgal biomass grown on a thin-layer photobioreactor treating wastewater was proposed carrying out and ex-ante economic and environmental analysis (*Chapter 8*) with the aim of studying the:

- Sizing and balances of a process to produce peptides and polyhydroxyalkanoates from algal biomass grown on wastewater.
- Economic (by the net present value, NPV) and environmental sustainability (by the global warming potential, kg CO_2 eq) of the proposed biorefinery processes.
- Weakness and critical points of the proposed process with possible improvements and research concerns to achieve a feasible process prior to scale-up.

CHAPTER 3: Agricultural products from algal biomass grown in piggery wastewater: a techno-economic analysis

3. AGRICULTURAL PRODUCTS FROM ALGAL BIOMASS GROWN IN PIGGERY WASTEWATER: A TECHNO-ECONOMIC ANALYSIS

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ABSTRACT

The intensification of livestock activities lead to an increase in waste generation with high content of nutrients like nitrogen and phosphorus, as is the case of piggery wastewater. However, this type of residue can be used as culture media for algae cultivation in thin-layer cascade photobioreactors to reduce its environment impact and produce a valorizable algal biomass. Biostimulants were produced by enzymatic hydrolysis and ultrasonication of microalgal biomass, using membranes (Scenario 1) or centrifugation (Scenario 2) as harvesting methods. The co-production of biopesticides by solvent extraction was also evaluated using membranes (Scenario 3) or centrifugation (Scenario 4). The four scenarios were analyzed by a technoeconomic assessment estimating the total annualized equivalent cost and the production cost, i.e., the minimum selling price. Centrifugation provided biostimulants approximately 4 times more concentrated than membranes, but with higher expense due to the cost of the centrifuge (contribution of 62.2% in scenario 2) and the electricity requirements. The biopesticide production resulted the highest contribution to investment cost in scenarios 3 and 4 (34% and 43% respectively). The use of membranes was also more advantageous to produce biopesticides, although it was 5 times more diluted than using centrifuge. The biostimulant production cost was 65.5 €/m^3 with membranes and 342.6 €/m^3 by centrifugation and the biopesticide production cost was 353.7 €/m^3 in scenario 3 and 2,122.1 €/m^3 in scenario 4. Comparing the treatment of 1ha of land, the cost of the biostimulant produced in the four scenarios was lower than the commercial one (48.1%, 22.1%, 45.1% and 24.2% respectively). Finally, using membranes for biomass harvesting allowed economically viable plants with lower capacity and longer distance for biostimulant distribution (up to 300 km) than centrifuge (188 km). The algal biomass valorization for agricultural products production is an environmentally and economically feasible process with the adequate capacity of the plant and distribution distance.

CHAPTER 4: Life cycle assessment of biostimulant production from algal biomass grown on piggery wastewater

4. LIFE CYCLE ASSESSMENT OF BIOSTIMULANT PRODUCTION FROM ALGAL BIOMASS GROWN ON PIGGERY WASTEWATER

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ABSTRACT

Piggery wastewater has become a large source of pollution with high concentrations of nutrients, that must be managed and properly treated to increase its environmental viability. Currently, the use of microalgae for treating this type of wastewater has emerged as a sustainable process with several benefits, including nutrient recovery to produce valuable products such as biostimulants, and CO_2 capture from flue gases. However, the biostimulant production from biomass grown on piggery wastewater also has environmental impacts that need to be studied to identify possible hotspots. This work presents the life cycle assessment by IMPACT 2002+ method of the production of microalgae-based biostimulants, comparing two different harvesting technologies (membrane in scenario 1 and centrifuge in scenario 2) and two different technologies for on-site CO_2 capture from flue gases (chemical absorption and membrane separation). The use of membranes for harvesting (scenario 1) reduced the environmental impact in all categories (human health, ecosystem quality, climate change, and resources) by 30% on average, compared to centrifuge (scenario 2). Also, membranes for CO_2 capture allowed to decrease environmental impacts by 16%, with the largest reduction in the resource category (~33%). Thus, the process with the best environmental viability was achieved in scenario 1 using membranes for CO_2 capture, with a value of 217 kg CO_2 eq/ha of crops. In scenario 2 with centrifugation, the high contribution of the cultivation sub-unit in all impacts was highlighted (>75%), while in scenario 1 the production sub-unit also had moderate contribution in the human health (~35%) and climate change (~30%) categories due to the lower concentration and high flow rates. These results were obtained under a worst-case situation with pilot scale optimized parameters, with limited data which would have to be further optimized at industrial-scale implementation. The sensitivity analysis showed a little influence of the parameters that contribute the most to the impacts, except for the transportation of the piggery wastewater to the processing plant in scenario 2. Because of the relevant impact of biostimulant transportation in scenario 1, centrifugation becomes more favourable when transportation distance is longer than 321 km.

CHAPTER 5: Effect of process parameters on the valorization of components from microalgal and microalgal-bacteria biomass by enzymatic hydrolysis

5. EFFECT OF PROCESS PARAMETERS ON THE VALORIZATION OF COMPONENTS FROM MICROALGAL AND MICROALGAL-BACTERIA BIOMASS BY ENZYMATIC HYDROLYSIS

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ABSTRACT

Photobioreactors for wastewater treatment coupled with nutrient recovery from the biomass is a promising biorefinery platform but requires working with microalgae-bacteria consortia. This work compares the effect that hydrolysis time and different enzymes have on the solubilization and recovery of components from microalgae bacteria grown in piggery wastewater and microalgae grown in synthetic media by enzymatic hydrolysis. Higher carbohydrate solubilizations were obtained from microalgae-bacteria than from pure microalgae (38.5% vs. 27% Celluclast, 5 h), as expected from the SEM images. Proteases solubilized xylose remarkably well, but xylose recovery was negligible in all experiments. Alcalase hydrolysis (5 h) provided the highest peptide recovery from both biomasses (≈34%), but the peptide sizes were lower than 10 kDa. Low peptide recoveries (<20%) but larger peptide sizes (up to 135 kDa) were obtained with Protamex. Pure microalgae resulted in remarkably higher losses, but similar amino acid profiles and peptide sizes were obtained from both biomasses.

CHAPTER 6: Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass

6. ASSISTED-ENZYMATIC HYDROLYSIS VS CHEMICAL HYDROLYSIS FOR FRACTIONAL VALORIZATION OF MICROALGAE BIOMASS

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ABSTRACT

Despite the interest in the utilization of photobioreactors as an alternative wastewater treatment, the research about posterior recovery and valorization of nutrients accumulated in the biomass is still limited. This work compared several hydrolysis methods for the recovery of proteins and carbohydrates from the biomass grown on a photobioreactor treating swine wastewater. Ultrasound-assisted and microwave-assisted enzymatic hydrolysis at mild conditions and chemical methods at different temperatures (40, 60, 120ºC) were applied to the microalgae and bacteria biomass. Alkaline hydrolysis provided the greatest peptide recoveries, increasing with temperature up to a maximum of 81%, but with very small peptide sizes in all temperature range. Acid hydrolysis provided the highest carbohydrate recoveries (60.7% at 120ºC) but degraded proteins, even at mild temperatures. Protein degradation did not vary with temperature in each chemical hydrolysis, obtaining similar peptide sizes in all temperatures, while carbohydrate losses were higher at lower temperatures. Ultrasound-assisted enzymatic extraction recovered 43.6% of the initial proteins as large peptides (up to 135 kDa) with the highest peptide purity (46.7%). Microwave-assistance increased the carbohydrate solubilization of enzymatic hydrolysis, achieving yields of 73% of xylose, but with significant losses.

CHAPTER 7: Effect of veterinary antibiotics and heavy metals on the composition and valorization of a consortium of microalgae and bacteria

7. EFFECT OF VETERINARY ANTIBIOTICS AND HEAVY METALS ON THE COMPOSITION AND VALORIZATION OF A CONSORTIUM OF MICROALGAE AND BACTERIA

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ABSTRACT

Piggery wastewater treatment with microalgae is a promising process which allows to produce valuable biomass, although the presence of antibiotics or heavy metals can influence the biomass composition and the valorization processes. This research studies the effect of doping the feed of a 1,200L photobioreactor treating pig manure with 1) veterinary antibiotics; 2) copper, zinc, and arsenic; and 3) combination of both pollutants. The pollutants presence decreased glucose content by up to 42% and increased protein and xylose by 30% and 16%. The pollutants increased the protein solubilization by acid hydrolysis at 120ºC by 32% while reduced glucose solubilization by 49% after alkaline hydrolysis at 120ºC. Applying enzymatic hydrolysis and ultrasound assisted enzymatic extractions, glucose and xylose recoveries were drastically reduced in presence of heavy metals (~100%). Antibiotics increased xylose solubilization by ultrasonication (74%), but also its degradation, decreasing xylose recovery. Doped metals were found in the chemical hydrolyzates (>60%).

KEYWORDS

Algal biomass, carbohydrates, piggery wastewater, proteins, hydrolysis.

1. INTRODUCTION

Pig manure has become a huge source of environmental pollution (Li et al., 2020) that must be treated to prevent contamination. This type of residue is rich in nutrients like organic matter (2,000 – 30,000 mg/L), nitrogen (200 – 2,055 mg/L), and phosphorus (100 – 620 mg/L) (Rojo et al., 2023b) that must be eliminated prior to discharge. However, the concern about the presence of other microcontaminants as certain veterinary antibiotics (used to treat and prevent animal diseases) and heavy metals (present in well water or/and used in animal feed as micronutrients) in the pig manure has increased in the last years (López-Serna et al., 2019) due to the severe environmental and health problems associated with them. Veterinary antibiotics (VA) are emerging pollutants used in farms and they are poorly absorbed by pig's digestive tract, hence between 44 – 72% of the drugs administered are being excreted in the manure (Conde-Cid et al., 2020), achieving concentrations between 0.01 and 100 mg/L (López-Serna et al., 2019). Different types of antibiotics are used in pig farms and found in pig manure including sulphonamides (such as sulfadiazine), fluroquinolones (such as ciprofloxacin) and tetracyclines (López-Serna et al., 2019; Nagarajan et al., 2019; Zambrano et al., 2023). On the other hand, heavy metals (HM) are usually found in liquid pig manure because animal feed contains traces of these elements, including copper and zinc (Wang et al., 2023), which are essential micronutrients in animal growth. Arsenic can also be found because it is present in the well water. All these elements can be found in pig manure with usual concentrations between 4.7 – 148 mg/L (copper), 12 – 234 mg/L (zinc), and <690 µg/L (arsenic) (Collao et al., 2022).

Both types of contaminants cannot be removed by conventional wastewater treatment plants (Amaro et al., 2023), and bioremediation with microalgae has emerged as a promising technology in terms of efficiency and potential use of generated biomass to produce high-added value products (Rempel et al., 2021a; Saavedra et al., 2019). VA removal mechanisms with microalgae can be divided into bio-adsorption (based on passive binding of the contaminants to the solid biomass), bio-accumulation (contaminants cross the cell wall membrane and they are assimilated by the microalgae cell), bio-degradation (break down of antibiotics to simple molecules by algae within or outside the cells) and photo-degradation by direct photolysis (Rempel et al., 2021b; Ricky and Shanthakumar, 2022). Likewise, heavy metals can be eliminated by bio-adsorption and, bio-accumulation since this type of biomass can bind the cellular

structure with these contaminants with high affinity (López-Pacheco et al., 2021; Saavedra et al., 2019). These elements react with proteins, lipids, and carbohydrates in the external cell wall, as proteins consist of amino acids that have metal binding groups and polysaccharides in the cell wall provides carboxy, sulphate, and amino groups (Pavithra et al., 2020).

After the bioremediation process, the produced biomass can be used to generate high-added value products, but it is possible that VA or HM would be present in this biomass and affect its macromolecular composition (Leong and Chang, 2020). As example, the carbohydrate content of *Chlorella* spp. grown on swine wastewater increased up to 52.7% from 40% while protein content decreased to 37% from 46% by addition of veterinary antibiotics such as tetracycline (1 mg/L) and doxycycline (1 mg/L) (Michelon et al., 2022). On the other hand, heavy metals favoured lipid accumulation, increasing its content in *Chlorella minutissima* with the addition of 0.4 mM of Cd^{2+} (21%) and 0.4 mM of Cu^{2+} (94%), but the protein content of *Chlorella vulgaris* decreased with the presence of cobalt (10^{-9} M), copper (10^{-9} M), and zinc (10^{-9} M) in the growth medium (Salama et al., 2019).

Nevertheless, there is very scarce research about the influence of these contaminants in microalgae biomass valorization processes. Rempel et al., (2021b) investigated the effect of various emerging pollutants (paracetamol, diazepam, fluoxetine, acetylsalicylic acid, and caffeine) on growth, chemical composition, and carbohydrate extraction applying only enzymatic hydrolysis (with 1% v/v of Liquozyme Supre 2.2X and AMG 300L) of various pure microalgae species (*Spirulina, Chlorella* and *Scenedesmus*) grown on synthetic media. Only acetylsalicylic acid and caffeine (with concentrations between 1 and 100 mg/L) affected the carbohydrates and proteins content in all species, but there was not influence of these two emerging pollutants in the carbohydrate extraction by enzymatic hydrolysis. Protein extraction was not investigated in this study. On the other hand, Tejirian and Xu (2010) studied the effect of ferric ions (0.25 M) on enzymatic hydrolysis of lignocellulosic materials with cellulases, finding decreases on recovery yields of up to 90%. It is demonstrated that HM can inhibit the activity of some enzymes, which would consequently influence the performance of an enzymatic hydrolysis process. Among them, Zn^{2+} can inhibit many protease enzymes due its strong interactions with aspartic acid, glutamic acid, and cysteine (Maret, 2013) while Fe ions or oxidative metal ions can inhibit cellulase

activity (Agrawal et al., 2021). Besides enzymatic hydrolysis, there are more extraction methods which could be influenced by VA and HM, including chemical, physical, or assisted enzymatic methods that have provided interesting results with this microalgae-bacterial residual biomass (Rojo et al., 2023a). Nevertheless, to the best of our knowledge, there is no research about their impact in these types of treatments.

This research is pioneer in the study of the influence of three VA (sulfadiazine, tetracycline, and ciprofloxacin) and three HM (copper, zinc, and arsenic) in the composition and extraction of proteins and carbohydrates from a microalgal-bacterial consortium grown on a 1,200 L thin-layer cascade photobioreactor fed with pig manure and doped with these two types of emerging pollutants. The extraction methods studied were chemical (acid and alkaline), enzymatic (with protease), ultrasounds and ultrasound-assisted enzymatic hydrolysis. Protein and carbohydrate solubilization from biomass were determined, along with peptide and monosaccharide recovery in the hydrolyzates. Composition and VA and HM content of the initial biomasses were analyzed, along with the cellular structure by scanning electron microscopy (SEM). Finally, the content of heavy metals in the hydrolyzates was also determined.

2. MATERIALS AND METHODS

2.1. Biomass cultivation conditions

Three assays were carried out in a thin-layer photobioreactor of 1,200 L working with a dilution rate of 0.2 d^{-1} fed with 10%-diluted piggery wastewater and inoculated with *Scenedemus almeriensis* located in IFAPA facilities in Almería (Spain). Three antibiotics commonly used on pig farms and belonging to different types of drugs (sulfadiazine (SDZ), tetracycline (TET), and ciprofloxacin (CIP)) were selected for VA doping experiments and copper, zinc and arsenic were added for HM doping experiments. The concentrations used in this study are shown in Table 1 based on typical values observed in this type of residue (Collao et al., 2022; López-Serna et al., 2019; Zambrano et al., 2023). Starting each assay, the photobioreactor was run for 15 days until the steady state was reached to obtain undoped biomasses (UB) as controls. Then, from day 16 to day 36, the feed was doped with three VA (SDZ, TET, CIP) in assay 1, three HM (copper, zinc, and arsenic) in assay 2 and both types of pollutants (3 VA and 3 HM) in assay 3, to obtain the doped biomasses (DB). More days were necessary to achieve the steady state in this period due to the stress conditions. More

days were planned to ensure steady state in the periods with doping, due to the stress on biomass growth caused by the presence of contaminants in the culture medium. After each assay, the photobioreactor was emptied and cleaned before starting the next experiment. Assay 1 was carried out in November with an average solar radiation of 11 MJ/m^2 (range between 9 and 13.2 MJ/m^2) and average daily temperatures ranging from 15 to 20ºC, assay 2 in March with an average solar radiation of 18 MJ/m^2 (range between 15.7 and 22.2 MJ/m^2) and average daily temperatures ranging from 15 to 16ºC and finally, assay 3 in May with an average solar radiation of 26 MJ/m^2 (range between 21.4 and 28.4 MJ/m^2) and average daily temperatures ranging from 18 to 23ºC.

	Pollutant	**Concentration**
Assay 1 (VA)	Sulfadiazine (SDZ)	100 µg/L
	Tetracycline (TET)	100 µg/L
	Ciprofloxacin (CIP)	100 µg/L
Assay 2 (HM)	Copper ($CuCl_2 \cdot 2H_2O$)	20 mg/L
	Zinc ($ZnCl_2$)	20 mg/L
	Arsenic ($Na_2HAsO_4 \cdot 7H_2O$)	30 µg/L
Assay 3 (VA and HM)	Sulfadiazine (SDZ)	100 µg/L
	Tetracycline (TET)	100 µg/L
	Ciprofloxacin (CIP)	100 µg/L
	Copper ($CuCl_2 \cdot 2H_2O$)	20 mg/L
	Zinc ($ZnCl_2$)	20 mg/L
	Arsenic ($Na_2HAsO_4 \cdot 7H_2O$)	30 µg/L

Table 1. *Concentrations of emerging contaminants and heavy metals in each assay.*

The biomass concentration of the photobioreactor was determined every day as total suspended solids. Biomass samples were harvested at the end of each period (in the days 15 and 36 respectively), centrifugated, and freeze-dried to obtain the UB and DB samples. These samples were analyzed to obtain its composition (nitrogen, protein, amino acid profile, carbohydrate, lipid, volatile solids, and ash) and were subjected to the hydrolysis methods described below (Section 2.2). Likewise, the biomass of assay 1 was analyzed for the VA content, the biomass of assay 2 was analyzed for the HM content and the biomass of assay 3 was analyzed for the VA and HM content.

2.2. Hydrolysis methods

Several hydrolysis methods were carried out to extract proteins and carbohydrates from the different biomasses (UB and DB) collected from the photobioreactor according to Rojo et al. (2023a), which operation conditions were described in the Table 2. In brief, chemical methods at 120ºC (NaOH 120 and HCl 120) were carried out in an autoclave at pressure of 1 bar using 2M NaOH and HCl respectively. These methods allow to achieve high solubilization yields from microalgae biomasses grown on piggery wastewater of up to 90% of carbohydrates with acid hydrolysis and >80% of proteins with alkaline hydrolysis (Martin Juárez et al., 2021; Rojo et al., 2023a). On the other hand, physical methods with ultrasounds (UAE and UAEE-P) were performed with an ultrasonic probe UIP1000hd (Hielscher Ultrasound Technology, Germany) at 50% of amplitude in a temperature-controlled jacketed vessel at 50ºC and pH of 6.5.

Extraction method	T (ºC)	pH	Enzyme	Concentration
Ultrasonic assisted extraction (UAE)	50	6.5	-	-
Ultrasonic assisted enzymatic extraction (UAEE - P)	50	6.5	Protamex	1:100 w/w
Enzymatic hydrolysis (HE – P)	50	6.5	Protamex	1:100 w/w
Alkaline hydrolysis (NaOH 120)	120	-	NaOH	2M
Acid hydrolysis (HCl 120)	120	-	HCl	2M

Table 2. *Operation conditions of the different extraction methods.*

Ultrasonication is considered an efficient technology which can achieve high extraction yields in short times without affecting the molecules properties due to the mild operation conditions (Zheng et al., 2021). Finally, enzymatic methods (UAEE-P and HE-P) were performed using Protamex as enzyme with a concentration of 1:100 $w/w_{dry\ biomass}$. This enzyme is an endo-protease that can solubilize proteins selectively providing peptides with promising sizes (Rojo et al., 2021), while the application of ultrasound in combination with enzymes can improve the extraction efficiency of enzymatic hydrolysis with short hydrolysis time (Rojo et al., 2023a). All the hydrolysis experiments were performed for 1 hour with a biomass concentration of 5 % ($w_{dry\ biomass}/w$) and working volumes of 250 mL.

After hydrolysis experiments, the biomass suspensions were centrifuged at 7,800 rpm for 10 min to separate the solid waste (solid fraction) and the hydrolyzate (liquid fraction). The solid fractions were freeze-dried for following analysis. Weights, total and

volatile solid, and nitrogen concentrations were determined in both fractions to check mass balances. Protein and carbohydrate content were determined in the solid waste fractions, while peptide, monosaccharide, and HM concentrations were analyzed in the hydrolyzates. However, VA concentrations were not analyzed in the hydrolyzates since the analytical methodology required in this type of matrix has not yet been developed. Finally, the initial biomasses of all assays were analyzed by scanning electron microscopy (SEM) to analyze the changes in cellular structure by the presence of VA and HM.

2.3. Analytical methods

Total suspended solids (TSS), total solid (TS) and volatile solid content (VS) were determined by a gravimetric method (Collao et al., 2021; Van Wychen and Laurens, 2016). Protein content in the initial biomasses and both fractions after hydrolysis experiments was determined using the Total Nitrogen Kjeldahl method according to Rojo et al. (2021) and applying a nitrogen-protein factor obtained from the amino acid profile of each used biomass. The total amino acid profile of the initial biomasses was analyzed by HPLC according to internal analytical protocol of the Instrumental Techniques Laboratory (LTI – UVa) described in Rojo et al. (2021). Carbohydrate content in the initial biomasses and solid waste fractions was determined as monosaccharides (glucose, xylose, cellobiose, and arabinose) after a concentrated acid hydrolysis with H_2SO_4, based on a NREL procedure (Van Wychen and Laurens, 2013). The monosaccharide concentrations in the liquid fractions were quantified by high-performance liquid chromatography (HPLC) using a Shimadzu LC-2050 (Japan), a refractive index detector RID-20A (Japan) with a Bio-Rad HPX-87H ion-exclusion column and external standards. Lipid content in the initial biomasses was determined using a modified protocol based on a chloroform-methanol 2:1 extraction (Lee et al., 2020). The VA content was analyzed by ultra-high performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS/MS) according to the method described in López-Serna et al. (2019). Copper (Cu) and zinc (Zn) content was analyzed by inductively coupled plasma spectrometry coupled with an optical emission spectrophotometer (ICP-OES) while arsenic (As) content was analyzed by inductively coupled plasma source mass spectrometer (ICP-MS) according to (Collao et al., 2022). For these HM analysis, initial biomass and solid fractions were previously hydrolyzed

with nitric acid (HNO_3) at 0.1M. Finally, electronic micrographs were taken using a Jeol JSM-820 scanning electronic microscope (SEM).

2.4. Calculations

The components protein and carbohydrate solubilization were calculated with the following Equation 1:

$$\text{Compound solubilization}\left(\frac{\text{g compound}}{100\text{ g biomass}}\right)=\left(\frac{M_{\text{initial biomass}}\cdot C_{\text{initial biomass}}-M_{\text{solid waste}}\cdot C_{\text{solid waste}}}{M_{\text{initial biomass}}}\right) \quad \text{Eq. 1}$$

where $M_{\text{initial biomass}}$ was the mass of the initial biomass (g), $C_{\text{initial biomass}}$ was the component mass content in the initial biomass (%), $M_{\text{solid waste}}$ was the mass of the solid residue after hydrolysis (g), and $C_{\text{solid waste}}$ was the component mass content in the solid residue after hydrolysis (%). On the other hand, component recovery as peptide and monosaccharides were calculated with Equation 2:

$$\text{Compound recovery}\left(\frac{\text{g compound}}{100\text{ g biomass}}\right)=\frac{M_{\text{hydrolyzate}}\cdot C_{\text{hydrolyzate}}}{M_{\text{initial biomass}}} \quad \text{Eq. 2}$$

where and $M_{\text{hydrolyzate}}$ was the mass of the hydrolyzate after hydrolysis (g), and $C_{\text{hydrolyzate}}$ was the component mass content in the hydrolyzate after hydrolysis (%). Finally, solubilization and recovery yields (%) were determined with equations 3 and 4:

$$\text{Compound solubilization yield (\%)}=\left(1-\frac{M_{\text{solid waste}}\cdot C_{\text{solid waste}}}{M_{\text{initial biomass}}\cdot C_{\text{initial biomass}}}\right)\times 100 \quad \text{Eq. 3}$$

$$\text{Compound recovery yield (\%)}=\left(\frac{M_{\text{hydrolyzate}}\cdot C_{\text{hydrolyzate}}}{M_{\text{initial biomass}}\cdot C_{\text{initial biomass}}}\right)\times 100 \quad \text{Eq. 4}$$

2.5. Statistical analysis

The differences amongst the mean compositions and yields were analyzed by the least significant difference test (LSD) at a confidence level of 95% using the software Statgraphics Centurion 19. All the analysis were performed by duplicate and the results were expressed as the mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Effect of VA and HM on biomass composition

The biomass concentration in the photobioreactor (g/L) decreased by the doping in the three assays. Specifically, the biomass concentration was reduced by 13%, 10% and, 35% in assays 1, 2 and 3 respectively. No remarkable differences on environmental

conditions were observed between cultivation periods in each assay. Therefore, the decrease in biomass concentration observed in all assays in the second period (during doping) could be attributed to oxidative stress of the contaminants. Likewise, the highest concentrations were determined in assay 3 with the highest solar radiation (2.7 g/L with UB), which was carried out at the time of the year with the lightest, while the lowest concentrations were observed in assay 1 with the lowest solar radiation (0.5 g/L with DB). Microalgae are able to remove VA and HM from a waste effluent by different mechanisms, so these emerging pollutants can subsequently be found in the final biomass (Leong and Chang, 2020). None of the analyzed VA and HM were detected in the UB of the three assays.

None of the analyzed VA and HM were detected in the UB of the three assays. In assay 1, where the photobioreactor was doped with three different VA, the obtained biomass achieved a CIP, TET and SDZ concentration of 74, 79 and 76 µg/g respectively by removing more than 80% of VA from the photobioreactor feed, which are similar results to those previously reported by Zambrano et al. (2021). On the other hand, in assay 2 doped with HM, the final biomass contained 20 mg/g of Cu, 16 mg/g of Zn and 20 µg/g of As. The HM removal efficiency in the photobioreactor was >80% for Cu and Zn, and 65% for As, similar values to 81% of Cu and Zn removal and 51% of As removal reported by Collao et al. (2022) working with CSTR open photobioreactors fed with piggery wastewater diluted at 5% v/v. Finally, in assay 3 the CIP, TET and SDZ content in the biomass was only 18, 1 and 2 µg/g respectively by removing 68% of SDZ from the photobioreactor feed. CIP and TET were not detected in the photobioreactor output, which together with the low concentration of VA in the biomass in comparison with assay 1, could indicate a higher degradation of these compounds in presence of HM. Finally, Cu, Zn and As content was 15, 14 mg/g and 21 µg/g with removal efficiencies in the photobioreactor higher than 85% for the three HM.

The Figure 1 shows the compositions (proteins, carbohydrates, lipids) of freeze-dried biomasses before (UB) and after the doping (DB) with veterinary antibiotics (VA) and heavy metals (HM) in each assay. The different biomass compositions in different assays are related to the time of the year in which each experiment was carried out. Therefore, high protein and low carbohydrate contents are excepted during winter operation, while high carbohydrate and low protein contents are obtained during spring operation (Martin Juárez et al., 2021).

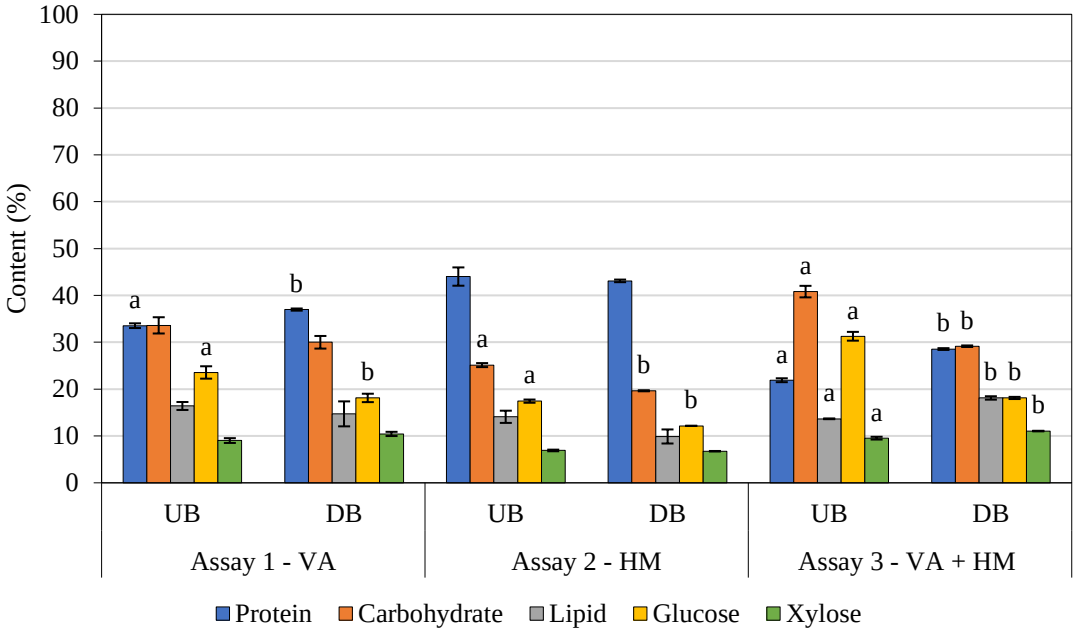


Figure 1. Biomass composition (ash-free dry basis). The data are provided as means ± standard deviations of 2 analytical determinations. Mean values with different letters are significantly different for $\alpha < 0.05$ by the LSD Test. UB = undoped biomass, DB = doped biomass.

VA had a slight influence on the microalgae biomass composition of the assay 1, with significant difference in protein and glucose content as reported by the LSD test ($\alpha < 0.05$). Protein content increased from 33.6% to 37%, while glucose content decreased from 23.6% to 18.1% in presence of these pollutants, while the decrease on lipids and the increase on xylose were not statistically significant. These changes produced by VA in the composition of the biomass could be attributed to the oxidative stress caused by the imbalance in the reactive oxygen species (Rempel et al., 2022; Wang et al., 2022). Michelon et al. (2022) investigated the influence of several VA (including tetracycline, oxytetracycline, chlortetracycline and doxycycline) on the composition of *Chlorella* spp. grown on piggery wastewater. Contrary to our findings, in this study protein content decreased from 45% to 37%, while the carbohydrate content increased from 40% up to 52.7% by the presence of 1 mg/L of tetracycline. However, Chen et al. (2020) showed that in the presence of 270 mg/L SDZ, the protein content of *Chlorella vulgaris* grown on synthetic media with BG-11 increased by 168% while carbohydrate content slightly decreased in presence of 30 mg/L of SDZ. Probably, the low VA concentration of our study (only 100 µg/L) compared to those used by Chen et al. (2020) resulted in lower change in protein content, although the same behavior to overcome the oxidative stress was observed.

Regarding the assay 2, the microalgae composition was also altered by the presence of heavy metals, obtaining significant differences by the LSD test ($\alpha < 0.05$) in carbohydrate and glucose percentages. In this case, the glucose content decreased from 17.4% to 12.2%, but the effect of heavy metals on protein, lipid and xylose contents was not statistically significant. This decrease in carbohydrate content by the presence of heavy metals have been attributed to oxidative stress and formation of reactive oxygen species which cause alterations of microalgae biological characteristics and damages in cell wall compounds and structure (Danouche et al., 2022; Piotrowska-Niczyporuk et al., 2012). The copper could inhibit the biosynthesis of the photosynthetic machinery, changing the microalgae composition (Aggarwal et al., 2011), while zinc could bind to thiols and led to intracellular metal accumulation and production of reactive oxygen species that damaged carbohydrates (Birben et al., 2012; Zhou et al., 2018).

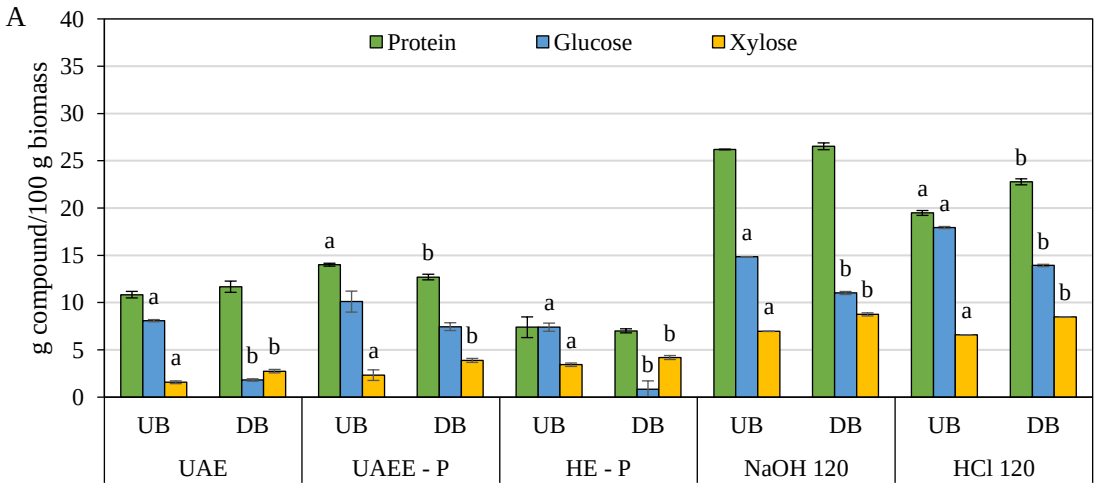
In the assay 3, the combined effect of VA and HM significantly changed all biomass macro components content (proteins, carbohydrates, lipids, and monosaccharides) as indicated by the LSD test ($\alpha < 0.05$). In this case, the protein, xylose, and lipid contents did increase by 30%, 16% and 33% respectively while glucose content decreased by 42%, resulting in 29% lower carbohydrate content. Therefore, the presence of both emerging pollutants had resulted in a big oxidative stress, causing an increase in protein synthesis to overcome the imbalance due to overproduction of reactive oxygen species (Chen et al., 2020; Wang et al., 2022) at the expense of the glucose content. Also, glucose synthesis may be inhibited by heavy metal toxicity (Aggarwal et al., 2011), while xylose shows the same behavior as proteins, to which it is associated in glycoproteins (Rojo et al., 2021). The lipid content also increased, but its low concentration compared to the rest of components, make it not worth studying. So, the stress generated by combining both types of pollutants greatly affected the composition of the doped algal biomass and was greater than when using these contaminants separately (assays 1 and 2).

The presence of the two types of contaminants also modified cell structure and morphology, as shown by scanning electron microscopy (SEM) of the different biomasses (UB and DB). In assay 1, the presence of veterinary antibiotics slightly increased the roughness of the cell wall compared to undoped biomass. Zambrano et al. (2021) studied the removal of different VA (including tetracycline, ciprofloxacin, and sulfadiazine) by a *Scenedesmus almeriensis* based microalgae-bacteria biomass and also

found a clear and similar alteration of the cell surface roughness. These changes were attributed to the binding of VA to different functional groups of the cell wall which altered the cellular structure. According to Zambrano et al. (2021), TET links with proteins, CIP with hydrogen bonds and SDZ with polysaccharides. Likewise, the presence of HM also varied the morphology of the biomass with rougher cell surface, attachment of cells by filaments and the presence of crystallized salts in the DB compared to UB. The same changes were observed by Urrutia et al. (2019), who studied the effect of heavy metals (Cu and Mo) on *Chorella vulgaris* morphology and found changes and damages in the morphology but also in the cell size due to the effect of the metal cations on membrane permeability which increased microalgae size in response to pressure (Geng et al., 2021). All these changes observed in the SEM images were a defense mechanism against antibiotic and metal stress to preserve algae cells from serious damage produced by these contaminants and protect internal content.

3.2. Effect of VA on protein and carbohydrate solubilization

Several types of hydrolysis were applied to the biomasses of the assay 1 (indicated in Table 2) for the extraction of proteins and carbohydrates, the principal macro-components of our microalgal biomass. All mass balances of nitrogen and volatile solids were determined, and very low losses were obtained, being always less than 7.7% and 4.2% respectively in all experiments. The amount of N supplied by the protease enzyme was considered negligible for all calculations in enzymatic treatments, since the enzyme/substrate ratio in the experiments was very low (Rojo et al., 2023a).



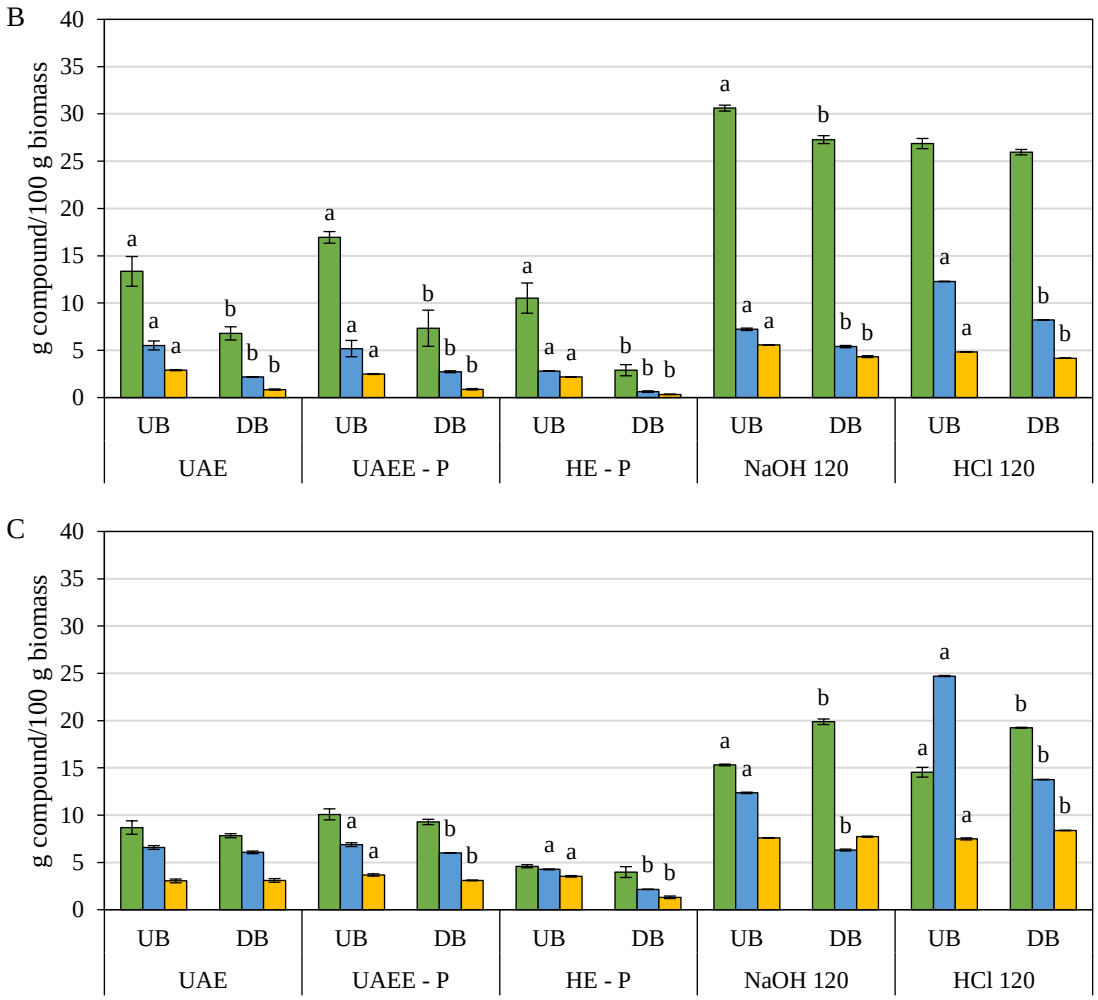


Figure 2. Protein solubilization ($g_{protein}$ /100 $g_{biomass}$), carbohydrate solubilization ($g_{carbohydrate}$ /100 $g_{biomass}$), glucose solubilization ($g_{glucose}$ /100 $g_{biomass}$) and xylose solubilization (g_{xylose} /100 $g_{biomass}$) in reference to the initial microalgal biomass in the assay 1 with veterinary antibiotics (A), assay 2 with heavy metals (B) and assay 3 with veterinary antibiotics and heavy metals (C). The data are provided as means ± standard deviations of 2 analytical determinations and the standard deviation of the means is represented by vertical interval lines. Different letters denote remarkable differences ($\alpha < 0.05$) according to LSD test. UB = undoped biomass, DB = doped biomass.

Figure 2.A shows the amounts of proteins and carbohydrates solubilized from undoped biomass (UB) and doped biomass (DB) after each hydrolysis treatment in the assay 1. As expected from previously published works (Martin Juárez et al., 2021; Rojo et al., 2023a), chemical hydrolysis with NaOH at 120ºC provided the highest protein solubilization, achieving 26.2 $g_{proteins}$/100 $g_{biomass}$ from UB and 26.5 $g_{proteins}$/100 $g_{biomass}$ from DB. The presence of VA did not significantly affect the protein solubilization by alkaline hydrolysis at 120ºC ($\alpha > 0.05$), but the difference in carbohydrate solubilization was significant according to LSD test ($\alpha < 0.05$). The doping of VA decreased glucose solubilization by alkaline hydrolysis from 14.9 $g_{glucose}$/100 $g_{biomass}$ (UB) to 11 $g_{glucose}$/100

$g_{biomass}$ (DB), while xylose solubilization increased from 6.9 g_{xylose}/100 $g_{biomass}$ (UB) to 8.7 g_{xylose}/100 $g_{biomass}$ (DB). Significant differences ($\alpha < 0.05$) were also found in terms of carbohydrate solubilization yields (decreasing from 69.9% to 66.6% for glucose but increasing from 86% to 92.2% for xylose), indicating possible structural changes in the cell that affected the extraction. Some VA, like tetracyclines are very sensitive to pH and at alkaline conditions, these compounds suffer an epimerization (Michelon et al., 2022) transforming into other compounds (iso-TC and 4-epi-iso-TC) but at acid conditions, the toxic compound 4-epianhydrotetracycline can be formed (Roy, 2011), which could explain why acid hydrolysis was most affected by the VA. However, alkaline hydrolysis is usually the most efficient method for extracting proteins (Rojo et al., 2023a), and the high efficiency of this treatment could override the VA effect.

On the other hand, as expected from previous works (Rojo et al. 2023a), chemical hydrolysis with HCl at 120ºC provided the highest carbohydrate solubilization, achieving 24.5 $g_{carbohydrates}$/100 $g_{biomass}$ from UB and 22.4 $g_{carbohydrates}$/100 $g_{biomass}$ from DB with significant differences ($\alpha < 0.05$). As occurred in the alkaline hydrolysis, glucose solubilization decreased in presence of VA (17.9 $g_{glucose}$/100 $g_{biomass}$ from UB and 13.9 $g_{glucose}$/100 $g_{biomass}$ from DB) while xylose solubilization increased (6.6 g_{xylose}/100 $g_{biomass}$ from UB and 8.5 g_{xylose}/100 $g_{biomass}$ from DB) with significant differences in both cases ($\alpha < 0.05$). The LSD test also found significant differences in xylose solubilization yields by acid hydrolysis, increasing from 81.4% with UB to 89.3% with DB. So, the presence of VA could also affect the effectiveness of this chemical treatment. High degradation of several emerging compounds (such as tetracycline or sulfonamides) is produced in acidic medium, decreasing their stability, and accelerating its degradation to produce other organic compounds (Hu et al., 2020; Michelon et al., 2022) which could influence the valorization process.

Regarding physical and biological methods, lower protein, and carbohydrate solubilization yields were obtained than by chemical treatments. According to the LSD test ($\alpha < 0.05$), the presence of VA only significantly influenced the protein solubilization by UAEE-P, reducing the amount solubilized from 14 $g_{proteins}$/100 $g_{biomass}$ to 12.7 $g_{proteins}$/100 $g_{biomass}$. Glucose solubilization decreased in presence of VA with significant differences according to the LSD test ($\alpha < 0.05$), especially after applying UAE (from 8.1 $g_{glucose}$/100 $g_{biomass}$ to 1.8 $g_{glucose}$ /100 $g_{biomass}$) and HE-P (from 7.4 $g_{glucose}$/100 $g_{biomass}$ to 0.9 $g_{glucose}$/100 $g_{biomass}$) while the combination of ultrasounds and

enzymes (UAEE-P) was able to extract this component (7.5 $g_{glucose}$/100 $g_{biomass}$) without significant differences according to the LSD test ($\alpha > 0.05$) between UB and BD. Significant differences were also detected ($\alpha < 0.05$) for xylose solubilization in all these physical and biological methods, slightly increasing this monosaccharide extraction with VA presence. The adsorption of SDZ and TET by the polysaccharides of the microorganism cells (Zambrano et al., 2021) could affect the macromolecular bindings, reducing the solubilization of glucose by mild methods (ultrasonication or enzymatic hydrolysis). However, the combination of ultrasounds and enzymes was able to break down the VA binds, allowing the glucose solubilization.

3.3. Effect of HM on protein and carbohydrate solubilization

As in the previous assay with VA, all mass balances of volatile solids and nitrogen were determined in the experiments carried out in the assay 2 with heavy metals. Low losses of nitrogen (analyzed again using the TKN method) and volatile solids were obtained, being always less than 7.2% and 6.5%.

Figure 2.B shows the solubilized amounts of proteins and carbohydrates by the different hydrolysis treatments in both microalgal biomasses (UB and DB) of assay 2. As in assay 1, chemical hydrolysis with NaOH at 120ºC provided the best results for protein solubilization, achieving 30.6 $g_{proteins}$/100 $g_{biomass}$ from UB and 27.3 $g_{proteins}$/100 $g_{biomass}$ from DB (co-solubilizing 7.2 $g_{glucose}$/100 $g_{biomass}$ and 5.1 g_{xylose}/100 $g_{biomass}$ from UB and 5.4 $g_{glucose}$/100 $g_{biomass}$ and 4.3 g_{xylose}/100 $g_{biomass}$ from DB). Thus, the presence of HM did influence protein and carbohydrate extraction by the alkaline hydrolysis at 120ºC as indicated by the LSD test ($\alpha < 0.05$). However, the same statistical analysis did not find significant differences in protein (~87%) and xylose (~90%) solubilization yields, while glucose solubilization yield increased with HM doping from 51.3% from UB up to 62.1% from DB, with significant differences ($\alpha < 0.05$). Therefore, the variations in protein solubilization could be due to the slight changes in the initial biomass composition, but the presence of HM produces structural changes by binding with functional groups of the cell wall. HM also induces the production of extracellular polymeric substances (EPS) which alter the cellular structure (Naveed et al., 2019). So, these changes possibly increase the availability of glucose, partly offsetting its lower concentration in the biomass.

On the other hand, chemical hydrolysis with HCl at 120ºC provided moderate carbohydrate solubilization with significant differences between UB and DB but solubilized similar amounts of proteins from both biomasses (26 $g_{proteins}$/100 $g_{biomass}$). As well as in the alkaline hydrolysis, the presence of HM decreased the amounts solubilized of glucose (from 12.3 $g_{glucose}$/100 $g_{biomass}$ to 8.2 $g_{glucose}$/100 $g_{biomass}$) and xylose (from 4.8 $g_{glucose}$/100 $g_{biomass}$ to 4.2 $g_{glucose}$/100 $g_{biomass}$). In terms of solubilization yields after acid hydrolysis, significant differences were only observed for glucose, increasing extraction values from 87.3% (UB) to 94.7% (DB). HCl has a high ability to desorb metals from biomass by damaging the metal-binding sites such as polysaccharides on the cell membrane surface (Manikandan et al., 2022). Again, the lower initial concentration of carbohydrates in the doped biomass (25.1% in UB vs 19.7% in DB) counteracts the increase on glucose solubilization yield by the presence of HM, resulting in an overall lower amount of solubilized carbohydrates from doped biomass.

About the presence of heavy metals in the hydrolyzates (Figure 3.A), which must be considered for further valorization processes, a significant percentage of the doped metals was found in the hydrolyzates from both chemical treatments. The highest difference between chemical treatments was the concentration of copper which is soluble in acid media but not in alkaline conditions. Therefore, 12% of the doped Cu was found in the alkaline hydrolyzate vs the 68.4% found in the acid hydrolyzate. Regarding the other heavy metals, higher amount of doped Zn was found in the acid (92.2%) than in the alkaline hydrolyzate (80.4%), but 81.9% of the doped As was detected in the alkaline hydrolyzate while 69.6% was found in the acid hydrolyzate. Therefore, the high chemical solubilization of heavy metals results in remarkable content of these elements in the chemical hydrolyzates and they must be considered for further valorization processes.

Regarding physical and biological treatments, the negative influence of HM on the extraction process of proteins and carbohydrates from the DB was very evident in all the experiments (confirmed by the LSD test with $\alpha < 0.05$). The amount of solubilized proteins decreased by the presence of HM after applying UAE (from 13.3 $g_{proteins}$/100 $g_{biomass}$ to 6.8 $g_{proteins}$/100 $g_{biomass}$), UAEE-P (from 16.9 $g_{proteins}$/100 $g_{biomass}$ to 7.3 $g_{proteins}$/100 $g_{biomass}$) and HE-P (from 10.5 $g_{proteins}$/100 $g_{biomass}$ to 2.9 $g_{proteins}$/100 $g_{biomass}$). The same behavior was observed in the solubilization of carbohydrates (glucose and

xylose), decreasing from 8.4 $g_{carbohydrates}/100\ g_{biomass}$ to 3 $g_{carbohydrates}/100\ g_{biomass}$ in the UAE, from 7.7 $g_{carbohydrates}/100\ g_{biomass}$ to 3.6 $g_{carbohydrates}/100\ g_{biomass}$ in the UAEE-P and from 5 $g_{carbohydrates}/100\ g_{biomass}$ to 1 $g_{carbohydrates}/100\ g_{biomass}$ in the HE-P. In all cases, significant differences were also found in protein and carbohydrate solubilization yields according to the LSD test ($\alpha < 0.05$), with HM reducing the yields of the extraction of proteins, glucose, and xylose.

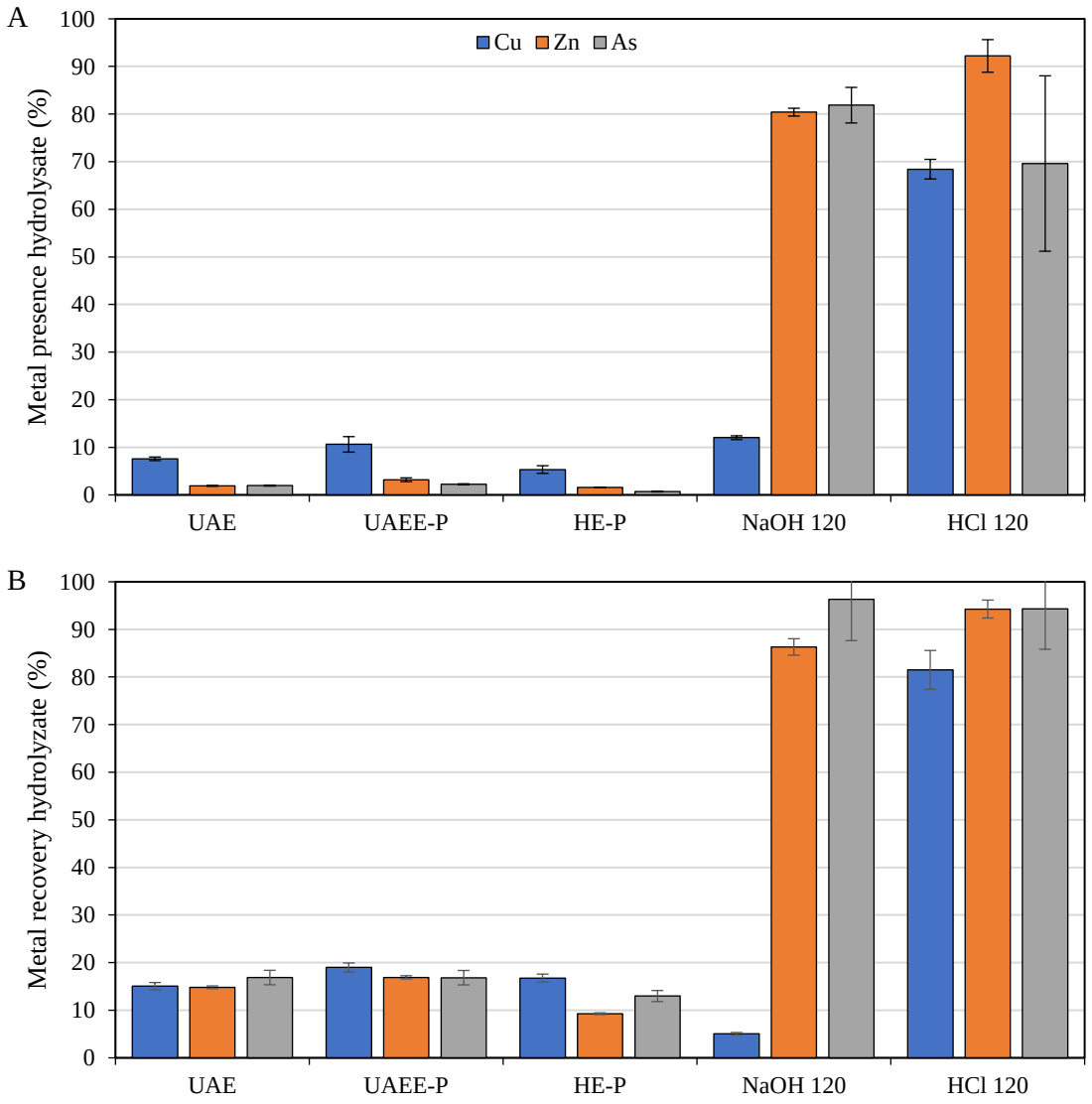


Figure 3. Heavy metal in hydrolyzate (%) in reference to the metal content in the initial microalgal biomass in the assay 2 (A) and assay 3 (B). The data are provided as means ± standard deviations of 2 analytical determinations and the standard deviation of the means is represented by vertical interval lines.

The decrease in yields of enzymatic treatments by the presence of HM was expected because enzymes are usually inhibited by the presence of heavy metals, among them zinc (Maret, 2013) and arsenic (Finnegan and Chen, 2012), which can bind to the thiol groups of protein enzymes, reducing the enzymatic activity. Also, the ions compete

for biding sites interfering the enzymatic activity (Smith et al., 2022). The same negative effect was observed by Tejirian and Xu (2010), who studied the enzymatic cellulose hydrolysis of pretreated corn stover (lignocellulosic biomass), obtaining also remarkable decrease on hydrolysis yields by the presence of some metal's ions (including Fe^{2+}, Cu^{2+} and Zn^{2+}).

The hydrolyzates of physical, and biological treatments contained small percentages of the doped elements (< 11%), showing that these methods did not remove heavy metal from biomass. In these cases, the highest solubilization was achieved for Cu, with values of 7.6%, 10.6% and 5.3% of the doped Cu in the UAE, UAEE-P and HE-P hydrolyzates respectively, showing how the application of ultrasound favored the Cu release (Geng et al. 2020).

3.4. Effect of VA and HM on protein and carbohydrate solubilization

All mass balances of volatile solids and nitrogen were determined in the experiments carried out in the assay 3 with both emerging pollutants. Low losses of nitrogen (analyzed again using the TKN method) and volatile solids were obtained, being always less than 9.8% and 8.7%.

Regarding both chemical treatments at 120ºC, significant differences were observed with the LSD test in the solubilization of proteins and carbohydrates ($\alpha < 0.05$) as shown in Figure 2.C. The amounts of proteins solubilized were lower with both, acid and alkaline, treatments from UB (15.3 $g_{proteins}/100\ g_{biomass}$ and 14.5 $g_{proteins}/100\ g_{biomass}$) than from DB (19.9 $g_{proteins}/100\ g_{biomass}$ and 19.2 $g_{proteins}/100\ g_{biomass}$). On the contrary, the glucose solubilization decreased significantly ($\alpha < 0.05$) from 12.4 $g_{glucose}/100\ g_{biomass}$ from UB to 6.3 $g_{glucose}/100\ g_{biomass}$ from DB for alkaline treatment and from 24.7 $g_{glucose}/100\ g_{biomass}$ from UB to 13.8 $g_{glucose}/100\ g_{biomass}$ from DB for acid treatment. No significant effect of doping on the amounts of xylose solubilized was found for alkaline treatment, while for acid hydrolysis, solubilization increased from 7.5 $g_{xylose}/100\ g_{biomass}$ (UB) to 8.4 $g_{xylose}/100\ g_{biomass}$ (DB). In terms of solubilization yields, significant differences were also found ($\alpha < 0.05$), but increasing with doping in all the cases, although only slightly for carbohydrates. The highest protein solubilization yield was obtained after alkaline hydrolysis from DB (82.6%) and the highest carbohydrate solubilization yield was obtained after acid hydrolysis from DB (90%). This behavior may be related to a high oxidative stress in presence of both emerging pollutants (Wang

et al., 2022) which reduced the membrane resistance to chemical treatment more than in the assays 1 and 2 for carbohydrate extraction. In the case of the proteins, to this increase in yield must be added their higher concentration in the initial doped biomass.

Concerning the presence of heavy metals in the hydrolyzates (Figure 3.B), a behavior like the assay 2 was observed, highlighting again the difference in the percentage of the doped Cu found in the hydrolyzate between acid (81.5%) and alkaline hydrolysis (5.1%). The highest amount of doped Zn in hydrolyzate was detected again after acid hydrolysis with 94.3%, but in this assay 3 the highest percentage of the doped As was also found in the acid hydrolyzate (94.3%).

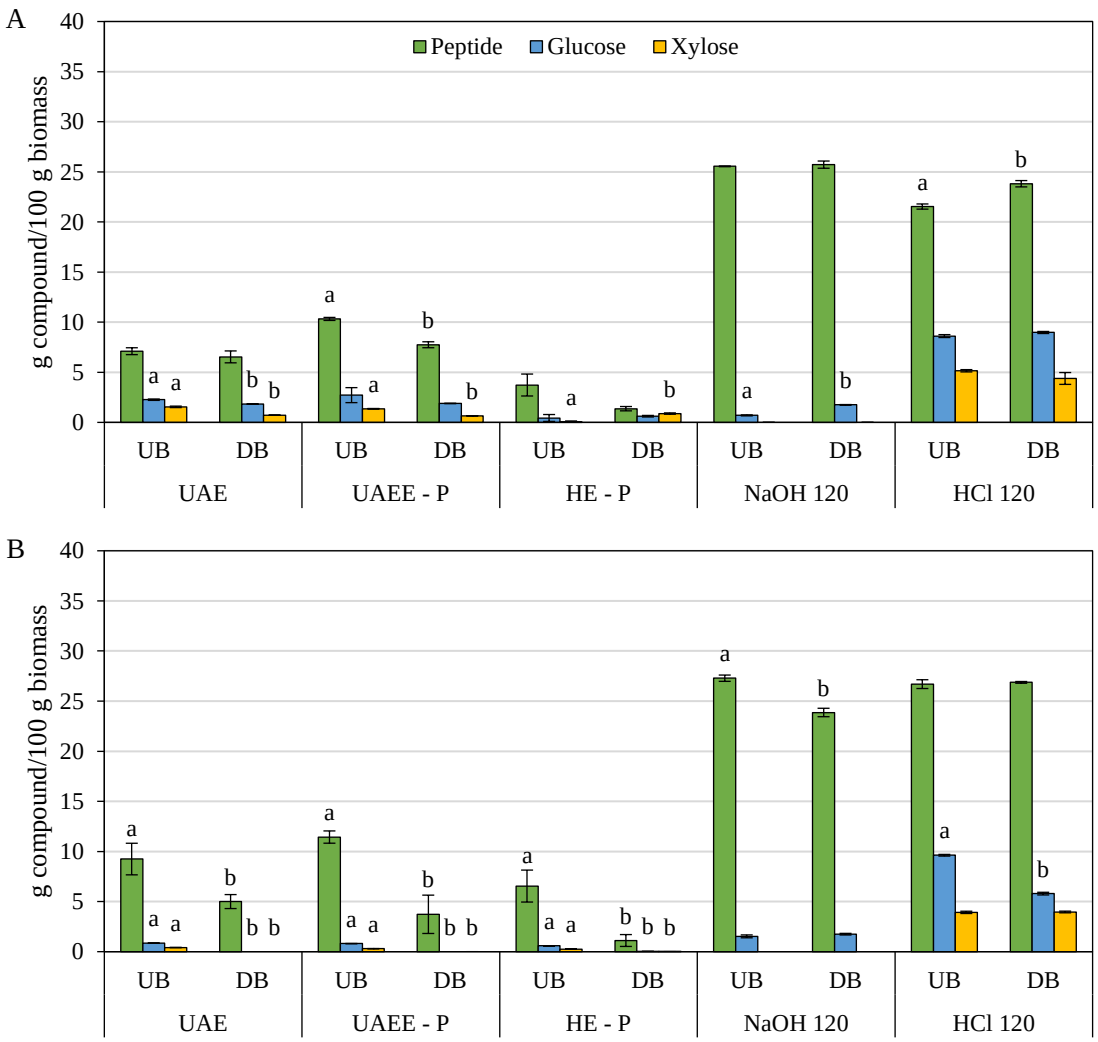
For physical and biological treatments, the amounts of solubilized proteins were not influenced by the presence of combined contaminants. Significant differences according to the LSD test ($\alpha < 0.05$) were found in carbohydrate solubilization after UAEE-P and HE-P treatments, decreasing the amount of solubilized glucose and xylose in presence of the contaminants (from 10.6 $g_{carbohydrates}$/100 $g_{biomass}$ to 9.1 $g_{carbohydrates}$/100 $g_{biomass}$ and from 7.8 $g_{carbohydrates}$/100 $g_{biomass}$ to 3.5 $g_{carbohydrates}$/100 $g_{biomass}$ respectively). Comparing the glucose solubilization yields, the only treatment affected by doping was UAEE-P, with an increase from 22.4% to 39.2%. The xylose solubilization yields decreased significantly after UAEE-P (from 40.6% to 33.2%) and after HE-P (from 39.7% to 13.6).

Finally, both physical methods (UAE and UAEE-P) and biological method (HE-P) provided similar presence of the 3 heavy metals in the final hydrolyzate (9.3 - 18.9%), being the highest value for Cu after UAEE-P and the lowest value for Zn after HE-P.

3.5. Effect of VA on peptide and monosaccharide recovery

After solubilization of the macro compounds, not all of them can be recovered since losses and degradation occur during the hydrolysis process. Figure 4.A shows the recovered amounts of peptides, glucose, and xylose from the biomass of assay 1 with VA. Chemical treatments at 120ºC achieved the highest recovery results (as for solubilization yields), highlighting the high peptide recoveries after alkaline treatment at 120ºC (~25 $g_{peptides}$/100 $g_{biomass}$ from UB and DB) and the high peptide (21.5 $g_{peptides}$/100 $g_{biomass}$ with UB and 23.8 $g_{peptides}$/100 $g_{biomass}$ with DB) and moderate glucose (~9 $g_{glucose}$/100 $g_{biomass}$) recoveries after acid treatment at 120ºC. Likewise, xylose was recovered after acid hydrolysis with final values around 5 g_{xylose}/100 $g_{biomass}$. Rojo et al.

(2023a) carried out acid and alkaline hydrolysis at 120ºC to microalgal biomass grown on piggery wastewater obtaining also high monosaccharide recovery yield (60.7% of initial carbohydrates) by acid and high peptide recovery yield by alkaline (81% of initial proteins) hydrolysis respectively. On the other hand, the LSD test ($\alpha < 0.05$) confirmed significant differences in the acid hydrolysis at 120ºC for the recovery of peptides, increasing by 6% with doped biomass. For both chemical methods, peptide losses during the hydrolysis process were similar from UB and DB, and different peptide recoveries by acid hydrolysis are related to different protein solubilizations. The very low recovery of carbohydrates by alkaline hydrolysis, reduces the relevance of the significant ($\alpha < 0.05$) increase in glucose recovery up to 1.8 $g_{glucose}/100\ g_{biomass}$ with doping for this treatment. After the alkaline hydrolysis no xylose was recovered, but after acid hydrolysis the xylose losses were significantly higher from doped biomass, so the presence of VA could promote the xylose degradation.



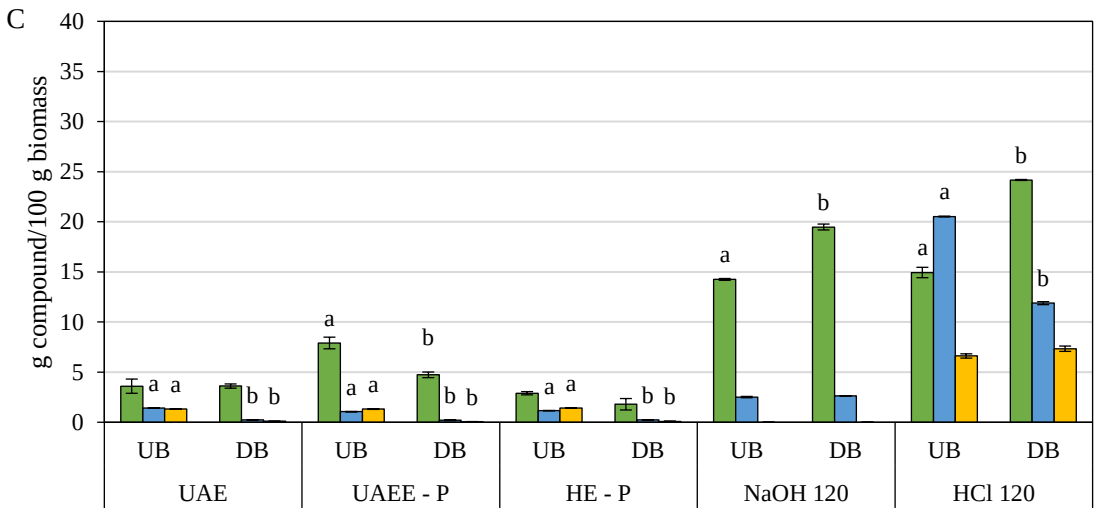


Figure 4. Peptide, glucose, and xylose recovery yield ($g_{compound}$ /100 $g_{biomass}$) in reference to the initial microalgal biomass in the assay 1 with veterinary antibiotics (A), assay 2 with heavy metals (B) and assay 3 with veterinary antibiotics and heavy metals (C). The data are provided as means ± standard deviations of 2 analytical determinations and the standard deviation of the means is represented by vertical interval lines. Different letters denote remarkable differences ($\alpha < 0.05$) according to LSD test. UB = undoped biomass, DB = doped biomass.

Regarding physical and biological methods, the recoveries were lower than for chemical methods, obtaining the highest peptide recovery of 10.3 $g_{peptides}$/100 $g_{biomass}$ with the UAEE-P from UB, followed by DB (7.8 $g_{peptides}$/100 $g_{biomass}$), UAE from UB (7.1 $g_{peptides}$/100 $g_{biomass}$) and from DB (6.5 $g_{peptides}$/100 $g_{biomass}$). A certain effect of the presence of VA on peptide losses could be observed in the UAEE-P method ($\alpha < 0.05$ according to the LSD test) since peptide recovery decreased in the DB by 25%, while solubilization decreased by only 9%. The same increase on peptide losses occurred in the UAE and HE-P treatment but without significant differences in peptide recovery according to the LSD test ($\alpha > 0,05$). On the other hand, glucose and xylose recoveries were reduced by doping with significant differences according to the LSD test ($\alpha < 0.05$) in some treatments, but again the low amounts of recovered monosaccharides (< 2.7 g/100 $g_{biomass}$) make a detailed analysis of these differences uninteresting. It is worth mentioning only the increase on xylose losses by the presence of VA, resulting on lower xylose recovery from DB than from UB, contrary to the effect of doping on xylose solubilization.

3.6. Effect of HM on peptide and monosaccharide recovery

Figure 4.B shows the recovery of peptides and the most abundant monosaccharides founded in the algal biomass (glucose and xylose) in assay 2. As can

be observed, again the chemical treatments at 120ºC provided the highest recoveries and more specifically, the alkaline achieved a recovered amount of 27.3 $g_{peptides}$/100 $g_{biomass}$ (UB), but with very low monosaccharides recoveries (< 2 g/100 $g_{biomass}$), while acid hydrolysis allowed for the recovery of high quantities of peptide (~26 $g_{peptides}$/100 $g_{biomass}$ from both biomasses) and moderate recoveries of glucose (9.6 $g_{glucose}$/100 $g_{biomass}$ from UB) and xylose (around 3.9 g_{xylose}/100 $g_{biomass}$). The LSD test ($\alpha < 0.05$) found significant effect of HM on peptide recovery after alkaline hydrolysis and on glucose recovery after acid hydrolysis. The decrease on peptide and glucose recoveries by heavy metal doping, with values of 23.9 $g_{peptide}$/100$g_{biomass}$ by alkaline treatment and 5.8 $g_{glucose}$/100$g_{biomass}$ by acid treatment was related to differences on solubilization. No effect of heavy metals on peptide and glucose losses was detected for chemical hydrolysis experiments.

Regarding physical and biological treatments, the notable influence that HM had on compounds recoveries can be observed in the Figure 3.B. The peptide recovery decreased in presence of these pollutants (significant differences, $\alpha < 0.05$), from 9.2 to 5 $g_{peptides}$/100 $g_{biomass}$ with UAE, from 11.4 to 3.8 $g_{peptides}$/100 $g_{biomass}$ with UAEE and from 6.6 to 1.1 $g_{peptides}$/100 $g_{biomass}$ with HE-P. No effect of HM on peptide losses was found, being these differences related to the effect of HM on solubilization, attributed to a possible enzyme inhibition (Finnegan and Chen, 2012; Maret, 2013). Finally, the recoveries of both monosaccharides were very low in all these experiments, being lower than 1 g/100$g_{biomass}$ or even null in the physical and biological treatments of DB and with significant differences due to HM doping ($\alpha < 0.05$) in the recoveries of glucose and xylose. The glucose and xylose losses in UAE, UAEE-P and HE-P treatments were higher from DB than from UB, which, together with the lower solubilization, resulted in a remarkable decrease in the amount of recovered monosaccharides due to the presence of HM.

3.7. Effect of VA and HM on peptide and monosaccharide recovery

The recoveries of peptides, glucose, and xylose of the assay 3 are illustrated in the Figure 4.C. The best results were achieved again by the chemical treatment with acid at 120ºC, with significant differences between undoped and doped biomass according to the LSD test ($\alpha < 0.05$) for peptides and glucose. The presence of the contaminants increased the peptide recovery from 14.9 $g_{peptides}$/100 $g_{biomass}$ from UB to 19.2 $g_{peptides}$/100 $g_{biomass}$ from DB and decreased the glucose recovery from 20.5 $g_{glucose}$/100

$g_{biomass}$ from UB to 11.9 $g_{glucose}$/100 $g_{biomass}$ from DB. These variations are similar to those found in the solubilization values (increase of ~30% for proteins and reduction of ~43% for glucose), indicating that there was no effect of the contaminants on peptides and glucose losses in the acid hydrolyzates. In the case of alkaline hydrolysis at 120ºC, a significant effect ($\alpha < 0.05$) of VA and HM on peptide recovery was observed, increasing from 14.3 $g_{peptides}$/100 $g_{biomass}$ from UB to 19.5 $g_{peptides}$/100 $g_{biomass}$ from DB. The difference between UB and DB peptide recovery in alkaline hydrolysis would be again due to the differences in solubilization which had similar percentage increases.

Regarding physical and biological treatments, the presence of VA and HM had a significant influence on the recoveries of peptides, glucose, and xylose (confirmed by the LSD test, $\alpha < 0.05$) except for peptides in the UAE and HE-P. Peptide recovery after UAEE-P achieved 7.9 $g_{peptides}$/100 $g_{biomass}$ from UB, and 4.7 $g_{peptides}$/100 $g_{biomass}$ from DB, decreasing by a higher percentage than the solubilization values shown above (8%). The presence of VA and HM could promote the degradation of solubilized proteins, decreasing the efficiency of the UAEE-P treatment. On the other hand, the presence of VA and HM also decreased the monosaccharide recoveries achieving very low recoveries (0.24 $g_{monosaccharide}$/100 $g_{biomass}$), highlighting the almost null values of xylose recovery in the three treatments. The presence of contaminants reduced monosaccharide recovery yields to a greater extent than solubilization yields. The combination of VA and HM favored the degradation of the released monosaccharides in UAE, UAEE-P and HE-P hydrolyzates.

4. CONCLUSIONS

The presence of veterinary antibiotics and Cu, Zn and As increased the protein and decreased the carbohydrate contents of microalgal biomass grown on piggery wastewater. Biomass concentration in the photobioreactor also diminished due to stress by both pollutants. Protein solubilization and recovery increased in presence of VA and/or HM after chemical treatments but decreased after ultrasound and enzymatic treatments. Glucose solubilization was greatly reduced by VA and HM for all the studied treatments. The effect of contaminants on xylose solubilization was similar than on proteins, but they increased degradation reducing its recovery. Finally, high percentage of doped metals was found in the hydrolyzates of chemical treatments compared to physical and biological methods (<20%).

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CHAPTER 8: Ex-ante and environmental assessment for microalgae valorization of biomass grown on wastewater treatment photobioreactor

8. EX-ANTE ECONOMIC AND ENVIRONMENTAL ASSESSMENT FOR MICROALGAE VALORIZATION FROM OF BIOMASS GROWN ON WASTEWATER TREATMENT PHOTOBIOREACTOR

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ABSTRACT

Waste biorefinery has become a promising process to produce valuable products while reducing the environmental impact of wastes and the consumption of non-renewable materials. One potential feedstock is the microalgal biomass grown on wastewater treatment photobioreactors, with high protein content, but not suitable for human uses. This research proposes a process to produce peptides and polyhydroxyalkanoates from algal biomass grown on wastewater, conducting preliminary techno-economic and environmental assessments taking as a basis of calculation 10 m^3/d of algae (95% humidity), and identifying the hotspots of the base process to optimize. An investment cost of ~4,863,000 € was needed to start up the base bioprocess related to a total equipment cost of ~1,654,000 € with 65% spending on centrifuges (the most expensive equipment). Annual operation cost was ~619,000 € related to the high production cost of the microalgae biomass (~201,800 €/year) and high electricity (~115,600 €/year) and heating (~65,100 €/year) requirements. An economically feasible process was achieved with a net present value of ~1,420,000 € and payback period of 10.6 years, that can be improved by optimizing the founded hotspots of the process. The reduction of the number of centrifuges improved significatively the net present value by 141%, while the reduction in the biomass production cost to the minimum of 0.77 €/kg_{DCW}, increased the net present value by 91%. The optimization of the electricity necessities of the extraction with ultrasounds would enhance the net present value by 60% and the increase of polyhydroxyalkanoates content of microorganisms increased the net present value by 84%. The life cycle analysis showed a global warming impact of 294 kg CO_2 eq/m^3 biomass. Within this environmental analysis, electricity was the greatest source of impact with 50% of the total global warming although this impact would reduce by 15% with the optimization of the electricity requirements.

KEYWORDS

Bioplastics, life cycle analysis, microalgae, peptides, techno-economic assessment, waste biorefinery.

1. INTRODUCTION

The increase in world population has led in recent years to several global economic and environmental issues such as resource depletion for food, energy and materials, wastewater production, and greenhouse gas (GHG) emissions. It is expected that in 2050 the demand for food, energy and materials increases by 60%, 50% and 40% respectively from 2020 (Catone et al., 2021) and thus, the research for new green sustainable processes and renewable feedstocks is imperative to overcome these problems. One promising technology is the microalgal biorefinery thanks to the potential of this type of microorganism to obtain multiple valuable products (Malik et al., 2022) and its culture advantages over other biomasses: i) fast growth rate, ii) high tolerance to a variety of environmental conditions for growth (temperature, pH, salinity), iii) inability to compete with agricultural crops, iv) high surface area productivity and photosynthetic efficiency (Kumar et al., 2022; Siddiki et al., 2022). However, microalgal biorefinery is not currently a viable process due to high production costs, high nutrients and energy requirements, low availability of the internal biomolecules, low economic viability, and unknown environmental impact (Goswami et al., 2022; Okeke et al., 2022). The use of farm or urban wastewater as culture medium can increase the process viability, as it provides the necessary nutrients for biomass growth along with an efficient treatment of this wastewater (Rojo et al., 2023a). The produced biomass consists of a consortium of microalgae and bacteria composed mainly by proteins due to the high nitrogen content of the wastewater, with carbohydrates being the second component (Rojo et al., 2023b).

Despite its interesting composition, this biomass cannot be employed for human uses due to its wastewater culture and other alternative uses are being explored. The high content of proteins in the biomass grown on N rich wastewater makes it useful as a raw material for this high value-added molecule (Moldes et al., 2022). Peptides from hydrolyzed proteins can be used in industry for gelling, foaming and emulsifying properties (Geada et al., 2021; Kumar et al., 2022) and/or in agriculture as biostimulants with bioactive properties or for animal feed (Andreola et al., 2023). The production of bioplastics is also an interesting possibility for microalgae valorization. Polyhydroxyalkanoates (PHAs) are gaining in popularity as substitute of petroleum-based plastics thanks to the higher thermal stability, easier biodegradability, and use of renewable feedstocks (Rajendran & Han, 2022). PHAs are usually produced and

accumulated by microorganisms which use carbon as nutrient (Tan et al., 2022). This carbon source can be obtained from the carbohydrate fraction of microalgae and many published works have proofed the technical viability of this process (Beckstrom et al., 2020; Goswami et al., 2022). Although microalgae are also used to produce biodiesel, the low lipid content of biomass grown on these wastewaters makes it unfeasible (Rojo et al., 2021).

Most of the existing research about waste and microalgae valorization is focused on the production of only one product which usually results in inviable processes and the cascade concept is in the early stages of actual biorefinery research. Therefore, the application of a multiproduct approach (e.g., by producing peptides and PHAs sequentially) could improve the economic and environmental sustainability of these processes (Okeke et al., 2022). The main bottlenecks in a microalgal biorefinery are in the downstream processing which involves the extraction and fractionation of the different compounds (Malik et al., 2022). This is principally due to the resistance of the cell wall for intracellular content extraction (Moldes et al., 2022), the challenge of extract different metabolites without affecting other compounds (Malik et al., 2022) and the presence of multiple compounds in the extracted hydrolyzate (Amorim et al., 2020. Malik et al., 2022). Macro-components can be extracted from biomass by different hydrolysis methods (physical, chemical and, biological). In this solubilization step, the proteins are hydrolyzed into peptides and carbohydrates into monosaccharides and some of the solubilized components degraded to other by-products (Martin Juárez et al., 2021). High severity conditions provide almost complete biomass solubilization, while mild methods result in moderate solubilization yields but the recovery of high peptide sizes with interesting functional properties (Martin Juárez et al., 2021; Rojo et al., 2023b).

To identify the best biorefinery process in economic and environmental terms and the hotspots at an early stage, techno-economic analysis (TEA) and life cycle analysis (LCA) are two important tools that provide the details about cost, energy, and environmental impacts (Thomassen et al., 2019) for a comprehensive assessment of the bottlenecks of a proposed process (Mehariya et al., 2021). An early-stage assessment improves the efficiency of the research & development process (R&D) and allows for go/no go and investment prioritization decisions, helping to control prohibitively high economic and environmental costs before implementation of emerging technologies

(Mahmud et al., 2021). Up to now, most TEAs and LCAs on algal biorefinery are focused on biofuel production (Ubando et al., 2022) with few articles about the economic viability and environmental impacts of the production of peptides or PHAs (Beckstrom et al., 2020; Seghetta et al., 2016). To the best of our knowledge, none published research used biomass grown on wastewater and integrating the production and fractionation of these two bioproducts. Thus, this paper is pioneer in the development and study of a biorefinery process for multi-production of peptides and PHAs from algal biomass grown on wastewater, analysing its economic and environmental feasibility, and identifying the principal weaknesses of the process and the possible improvements at an early stage.

2. MATERIALS AND METHODS

This section briefly describes the proposed biorefinery base process, indicating the operational parameters and calculation bases used in the research. To evaluate the techno-economic feasibility of the sequential production of peptides and PHAs from algal biomass grown on wastewater, the following economic indicators were estimated: i) the net present value, ii) the internal rate of return and iii) the payback period, in function of the established selling price of both products and analysing the possible improvements. On the other hand, a life cycle assessment was conducted to determine the environmental impacts associated with the whole process along with technical aspect of the process which needs to be optimized to reduce these impacts (Marangon et al., 2021).

2.1. Process description

The proposed base process consists of the sequential production of peptides and PHAs from microalgae biomass grown on piggery wastewater. This residue was selected as raw material for this study because piggery wastewater is an abundant waste with a high nitrogen content that is a serious and increasing environmental problem, for which treatment in microalgae photobioreactors has proven to be effective (Rojo et al., 2023a). The block diagram is represented in Figure 1 and the summary of the operational parameters in Table 1. The whole process is divided into 6 different sub-units: ultrasonication extraction (UAEE), solid separation, peptide purification, acid hydrolysis, PHAs production, and PHAs purification.

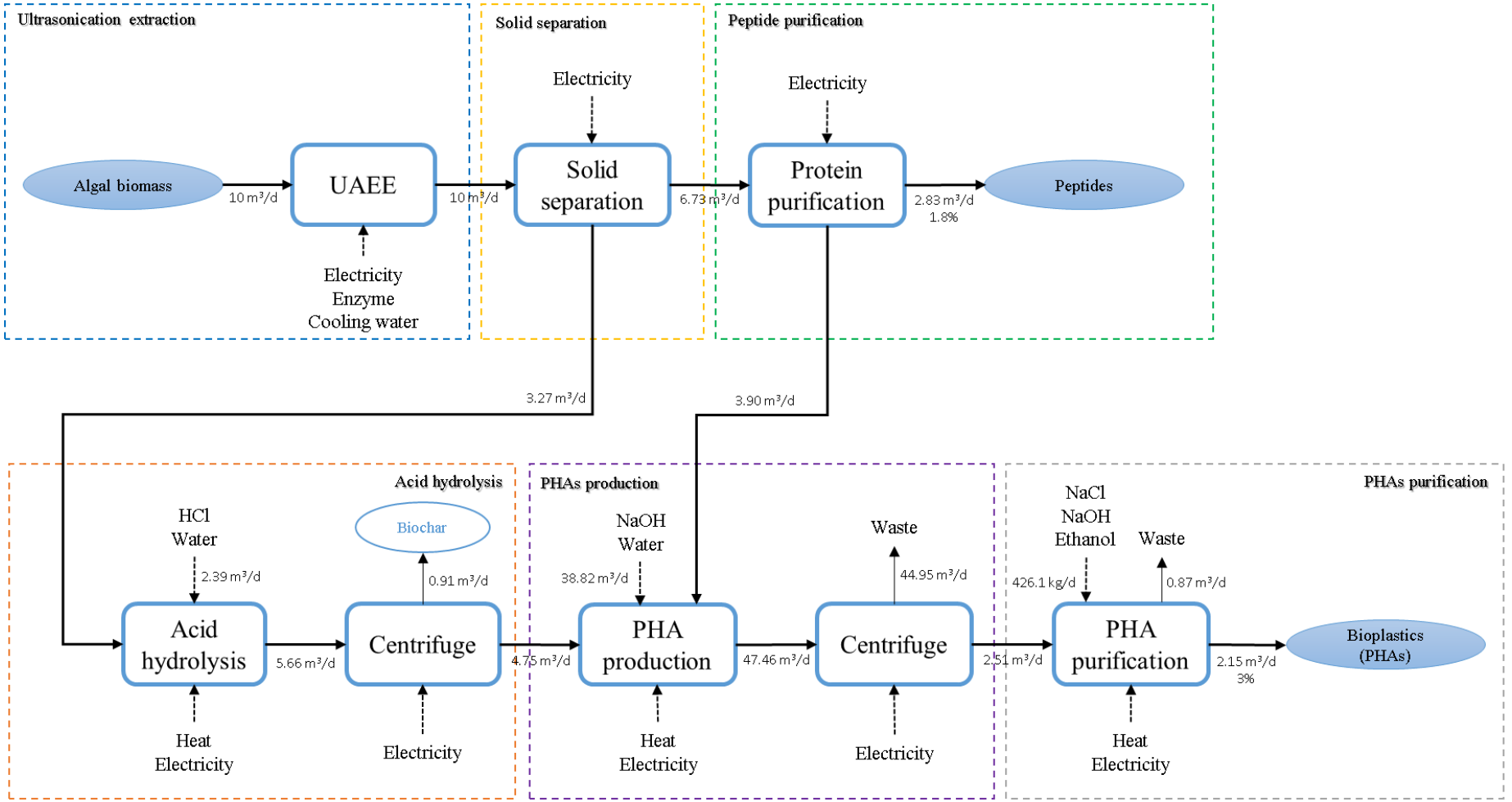


Figure 1. Process block diagram of the proposed biorefinery base process with sub-units and volumetric flows (m^3/d).

Sub-unit	Ultrasonication extraction (UAEE)	Solid separation	Peptide purification	Acid hydrolysis	PHA production	PHA purification
Operational parameters	T = 50ºC t = 1 hour [Enz] = 1% w_{CDW}/w Energy = 50 MJ/kg $S_{proteins}$ = 50% $S_{carbohydrates}$ = 46%	Centrifuge efficiency = 95% Solid concentration = 90 g/L Microfiltration efficiency = 100% Flux = 12.6 L/m^2/h	Ultrafiltration with PES membranes TMP = 1.1 bar Flux (50 kDa) = 27.4 L/m^2/h Flux (10 kDa) = 9.85 L/m^2/h Flux (5 kDa) = 8.15 L/m^2/h	T = 120ºC t = 1 hour [Solvent] = 2M [Biomass] = 5% w_{CDW}/w Centrifuge efficiency = 95% Solid concentration = 90 g/L $S_{proteins}$ = 76% $S_{carbohydrates}$ = 87%	10%-diluted acid hydrolyzate *Paracoccus denitrificans* T = 37ºC t = 36 hours Centrifuge efficiency = 95% Solid concentration = 90 g/L [Biomass] = 5 g/L PHA content = 30%$_{CDW}$	[NaCl] = 8 g/L [NaOH] = 4 g/L [Ethanol] = 20% v/v T = 30ºC t = 7 hours
Reference	(Rojo et al., 2023b)	(Liu et al., 2021; Rojo et al., 2023b)	(Trigueros et al., 2022)	(Rojo et al., 2023a)	(Abd El-Malek et al., 2022; Zhou et al., 2023)	(Anis et al., 2013)

Table 1. Summary of the operational parameters and conditions associated to each sub-unit of the proposed process.

2.1.1. Ultrasonication extraction (UAEE)

Microalgae biomass used in the base process was produced in a thin-layer cascade photobioreactor fed with diluted piggery wastewater (10% v/v) from two pig farms with ~3,200 heads located in the municipality of Cuéllar (Spain) (Rojo et al., 2023a). The total flow of microalgae suspension treated was 10 m^3/d with 95% of humidity after harvesting with a centrifuge (Rojo et al., 2023a). The dry biomass composition was 35.4% of proteins, 19.3% of carbohydrates (9.5% of glucose and 9.0% of xylose) and 6.1% of lipids (Rojo et al., 2021). This biomass was a consortium of microalgae and bacteria, being *Scenedesmus almeriensis* the major microalgae specie (96%) and the bacteria from the phyla *Proteobacteria*, *Firmicutes* and *Cyanobacteria* (Rojo et al., 2021).

This microalgae suspension was subjected to an ultrasonic assisted enzymatic extraction (UAEE) at 50ºC during 1 hour with Protamex as enzyme (1:100 w_{DCW}/w) and an applied ultrasounds energy of 50 MJ/kg. Hydrolysis method, solid biomass concentration, and operational conditions were selected according to the results of previous experimental research to obtain moderate protein solubilization, but low degradation of the targeted molecule (in our case, peptides) and high peptide sizes (Rojo et al., 2023b). The final solubilization of proteins and carbohydrates from the initial biomass considered in this study was 50% and 46% respectively. Likewise, degradation of proteins (11%) and carbohydrates (33%) also took place during the solubilization process, generating by-products useful for PHAs production. Therefore, the 39% of the initial proteins were recovered as peptides and 13% of the initial carbohydrates were recovered as monosaccharides in the hydrolyzate. All these solubilization, degradation and recovery yields refer to the amount of each component in the initial biomass. Finally, the obtained peptides were ranging from 11 kDa to 135 kDa in size (Rojo et al., 2023b).

2.1.2. Solid separation

After the extraction process with UAEE, the suspension was centrifugated (95% of efficiency) to obtain two different streams: i) 7.01 m^3/d of liquid phase (hydrolyzate) with the recovered peptides (9.9 g/L), monosaccharides (1.8 g/L), and other by-products (7.1 g/L) and, ii) 2.99 m^3/d of solid phase with the residual microalgae biomass at a concentration of 90 g/L (Rojo et al., 2023a). Centrifugation is commonly used to

separate protein-rich hydrolyzate from residual solid phase after extraction treatment (Rojo et al., 2022). However, this hydrolyzate stream had also an amount of residual solids (~2 g/L) which must be removed to improve the posterior peptide purification process. The elimination was carried out by microfiltration (MF), which achieved a 100% retention of the residual solids using a polyether sulfone (PES) membrane of 0.1 µm achieving a solid concentration in the retentate of 5%. The use of membranes to perform the elimination of residual solids has several advantages, including low energy consumption, continuous operation and easily up-scale (Zhao et al., 2023). Two different streams were finally obtained: i) 6.73 m^3/d of permeate stream with the peptide concentration of 9.9 g/L which have to be purified and, ii) 0.28 m^3/d of retained solids which will be combined with the flow of solids previously obtained in the centrifuge (2.99 m^3/d) and subjected to an acid hydrolysis.

2.1.3. Peptide purification

Peptide purification can be performed with several methods including chromatography, protein dispersion by precipitation (pH shift and salting out) and membrane technology (Amorim et al., 2020; Rojo et al., 2022). Regarding chromatography, this technology is based on the separation of peptides with a stationary phase based on their physical characteristics (molecular weight, electric charge, hydrophobicity…) (Alavi & Ciftci, 2023). On the other hand, protein dispersion is based on the protein precipitation by changing the pH to the isoelectric point (pH shift) or the addition of a salt to form aggregates (salting out) (Rojo et al., 2022). In this work, membrane technology was selected to purify the peptides due to the ability to preserve the functional properties of the hydrolyzed peptides comparing with others as protein precipitation. Also, it is a mature technology which requires low energy, can be easily scaled up with technical viability, operated in continuous mode and mild conditions, can be integrated into other chemical processes (Alavi & Ciftci, 2023) and it involves less amounts of additives of solvents and chemicals. So, the above final permeate stream from MF (6.73 m^3/d, 9.9 g/L of peptides, 1.8 g/L of monosaccharides and, 7.1 g/L of by-products) was subjected to ultrafiltration (UF) with membranes in series composed of polyether sulfone (PES) and pore size of 50 (Flux = 27.4 L/m^2/h), 10 (Flux = 9.85 L/m^2/h), and 5 kDa (Flux = 8.15 L/m^2/h) at a constant transmembrane pressure (TMP) of 1.1 bar (Trigueros et al., 2022) to finally obtain a stream of 2.83 m^3/d with a peptide concentration of 1.8% (Table 2).

2.1.4. Acid hydrolysis

On the other hand, the residual fraction from the solid separation sub-unit (3.27 m^3/d, 8.7% of solids) was subjected to an acid hydrolysis to hydrolyze the remaining cellular components as organic compounds which will be used as source of nutrients to cultivate microorganisms able to produce PHAs. This chemical treatment is based on the use of an acid (HCl) combined with relatively high temperatures (up to 120ºC) and allows to disrupt the microalgal cell wall with high efficiency and solubilization yields (Martin Juárez et al., 2021; Rojo et al., 2023b). It transforms the carbohydrates and proteins into monosaccharides, amino acids, organic acids, and other by-products assimilable by microorganisms (Tan et al., 2022). The operational conditions of the acid hydrolysis were 120ºC for 1 hour, 5% w_{DCW}/w and addition of hydrochloric acid (HCl) 2M, which allow to obtain high solubilization yields of proteins and carbohydrates (76.2% and 86.7% respectively) and high recovery yields of peptides and monosaccharides (61.5% and 60.7% respectively) (Rojo et al., 2023b). Also, by-products were generated from the carbohydrate's degradation (26.7%).

Although the main objective of the process is to analyze the production of PHAs and peptides from microalgal biomass grown on piggery wastewater, other product that can be profitable was also obtained in the acid hydrolysis unit (Table 2). The residual solid fraction from the acid hydrolysis which can be used as biochar (0.91 m^3/d and 9.5% of solids) a substitute of charcoal. In this way, a more economically sustainable process can be achieved by applying the concept of biorefining of multi-compound production (Moldes et al., 2022) by taking advantages of all the usable streams obtained.

2.1.5. PHAs production

The suspension obtained from the acid hydrolysis was centrifugated and the acid hydrolyzate (4.75 m^3/d) was neutralized. From the experimental results of our research group, a dilution of the acid hydrolyzate (10% v/v) is necessary to achieve a correct growth of the microorganisms since higher concentrations of nutrient or by-products inhibited it. Pure water and the monosaccharide solution from the peptide purification sub-unit (3.90 m^3/d, 1.8 g/L of monosaccharides and 7.1 g/L of by-products) are used to dilute the acid hydrolyzate. Thus, a final stream of 47.5 m^3/d feeds a stirred reactor (CSTR) where the PHAs-producing microorganisms will be cultured at 37ºC for 36

hours. The bacteria *Paracoccus denitrificans* was selected because it is able to accumulate PHAs using different carbon sources such as monosaccharides. acids and other organic compounds (glycerol, methanol, ethanol, …) (Mota et al., 2019) obtained from the acid hydrolysis of microalgae residual biomass. This type of organisms can grow up to 5 g/L and produce PHAs at a rate of 30%$_{CDW}$ (Abd El-Malek et al., 2022; Zhou et al., 2023), values that have been used in the proposed base process. The working pH was 7, reached by NaOH neutralization of the 10% diluted acid hydrolyzate which provided the compounds necessary for its growth (without supplementation with micronutrients). After the cultivation, the biomass suspension was again centrifugated (95% efficiency and 90 g/L of concentration) and the produced biomass (2.51 m^3/d) with intracellular PHAs was subjected to an extraction and purification process to separate PHA granules in the microorganism from non-PHA molecules (Kurian & Das, 2021). A waste liquid stream with low concentration of nutrients of the exhausted culture medium was also produced as residue (44.95 m^3/d).

2.1.6. PHAs purification

PHA extraction methods can be classified in two categories: i) digestion of non-PHA molecules using chemicals, enzymes, or mechanical disruption and, ii) solvent-based extraction methods (Mondal et al., 2023). Solvent-based methods are currently the most applied for PHAs extraction although some solvents (specially chlorinated) have high environmental and human impact, so it is not recommended. Therefore, the use of non-chlorinated solvents or chemicals like alkali compounds (NaOH) are recommended as an alternative with lower operation costs than other extraction methods (Mondal et al., 2023; Rodrigues et al., 2022). In our proposed base biorefinery process, a two steps extraction, the first one with 8 g/L of NaCl, the second one with 4 g/L of NaOH followed by precipitation with 20% v/v of ethanol was employed (Anis et al., 2013). This separation was carried out in a CSTR at 30°C with times of 3 hour for NaCl and 1 hour for NaOH extraction. The solvent waste stream (0.87 m^3/d) containing the ethanol was separated by centrifugation (95% efficiency). A final solid stream of 2.15 m^3/d with a PHAs concentration of 3.0% was obtained (Table 2).

Product	Flow (m^3/d)	Concentration (%)	Selling price	Reference
Peptides	2.83	1.8	2 €/kg	(Andreola et al., 2023)
PHA	2.15	3.0	0.15 €/kg[a]	(Beckstrom et al., 2020; Tan et al., 2022)
Biochar	0.91	9.5	0.01 €/kg[b]	(Martinez-Fernandez et al., 2021)
Raw material			**Price**	**Reference**
Enzymes	-	-	10 €/kg	(Rojo et al., 2023b)
Pure water	-	-	1.06 €/m^3	(Romero-García et al., 2022)
HCl	-	-	0.26 €/kg	(Pérez et al., 2021)
NaCl	-	-	0.07 €/kg	(Pérez et al., 2021)
NaOH	-	-	0.31 €/kg	(Pérez et al., 2020)
C_2H_6O	-	-	0.72 €/kg	(Pérez et al., 2020)
Cooling water			0.06 €/m^3	(Pérez et al., 2021)
Low pressure steam	-	-	0.15 €/kg	(Rojo et al., 2023b)

[a]*Determined based on the final concentration of PHA (5 €/kg).*
[b]*Determined based on the final solid concentration of biochar (87 €/T).*

Table 2. Products obtained during the biorefinery process (flows and concentrations), proposed selling prices based on the provided references and raw material prices.

2.2. Techno-economic assessment (TEA)

An economic assessment was performed using as a design basis, a biorefinery plant with and a lifetime of 15 years (Acién Fernández et al., 2019; Thomassen et al., 2019) and a treatment capacity of 10 m^3/d of microalgae biomass with 95% of humidity (section 2.1.1). A biomass price of 1.5 €/kg_{DCW} was considered using piggery

wastewater as nutrient source and a thin-layer cascade photobioreactor harvested with centrifuge (Rojo et al., 2023a; Romero-García et al., 2022). Likewise, the benefit of the pig manure treatment with microalgae was also considered, with a price of 2 €/m^3 of pig manure.

To evaluate the economic feasibility of the proposed processes, the net present value (NPV), the internal rate of return (IRR), and the payback period (PP) were calculated based on the selling prices established in Table 2 for all the obtained products (peptides, PHAs, and biochar). The NPV determined whether the investment can be recovered based on the cash flow across the lifetime, and was calculated with equation 1 (Giraldo et al., 2020; Orive et al., 2021):

$$NPV = \sum_{t=1}^{n} \frac{CF_t}{(1+r)^t} - IC \qquad \text{Eq. 1}$$

where CF_t is cash flow (Total income – total cost) for time t (€), IC is the total investment cost (€), r is the discount rate of 5% (Rojo et al., 2023a), and n is the lifetime of the project (15 years). A positive NPV value means the investment produces income above the operational costs and investment costs and therefore, the project is economically feasible (Giraldo et al., 2020). On the contrary, if NPV is negative indicates that total costs (investment costs and operational costs) are larger than total income and thus, the project is not feasible from an economic perspective. On the other hand, the IRR calculated with equation 2 was the discount rate which makes NPV = 0 and therefore, allows comparing the profitability among portfolio projects (Orive et al., 2021):

$$NPV = 0 = \sum_{t=1}^{n} \frac{CF_t}{(1+IRR)^t} - IC \qquad \text{Eq. 2}$$

Finally, the PP was defined as the time needed to recover the initial investment in terms of profits or savings (Coker, 2007; Giraldo et al., 2020) and corresponds to the year from which the cash flow becomes positive.

2.2.1. Investment costs (IC)

Investment costs (IC) included the total equipment cost (TEC), the fixed capital and the fix capital per year (Rojo et al., 2023a). The TEC for UAEE, solid separation, peptide purification, acid hydrolysis, PHAs production, and PHAs purification sub-units of Figure 1 was determined based on equipment costs reported in ASPEN HYSYS V.12

considering the inflation rate until 2023 in United States of America (13.5 %). The fixed capital and the fix capital per year were then calculated according to Lang's Factor method considering a solid-liquid process (Green & Perry, 2008; Rojo et al., 2023a).

2.2.2. Operational and maintenance costs (OMC)

OMC included the raw material necessities (which was determined based on the mass balances), energy requirements of the different equipment, and labor. The raw material required for the proposed processes included enzymes, pure water for dilution, HCl, NaCl, NaOH, ethanol, cooling water, and low-pressure steam. All prices were indicated in Table 2 and updated to 2023 € according to the inflation in Spain. On the other hand, energy requirements were established as 8 kWh/m^3 for the centrifuge (Zhao et al., 2023), 0.2 kWh/m^3 for reactor mixers, and 0.2 kWh/m^3 for membrane filtration (Rojo et al., 2023b) considering an electricity price of 0.15 €/kWh as average of the first trimester of 2023 (ESIOS 2023). Energy requirements for pumps was determined with equation 3 (Pérez et al., 2021):

$$P_{pump}(kW) = \frac{Q \cdot \Delta P}{\mu} \qquad \text{Eq. 3}$$

where Q is the volumetric flow (m^3/s), ΔP is the pressure drop (kPa) and μ is the efficiency (75%).

2.3. Life cycle assessment (LCA)

A life cycle assessment (LCA) was performed in compliance with the international standards ISO 14040:2006 and ISO 14044:2006 to evaluate the environmental performance of the proposed process. It was divided into four main steps: i) goal and scope definition, ii) life cycle inventory (LCI), iii) life cycle impact analysis, and iv) interpretation of results. This is considered as the most established method to shed light on the environmental performances of a desired process (Rojo et al., 2024).

2.3.1. Goal and scope

The main goal of this research is to investigate the environmental sustainability of microalgae processing to produce peptides and PHAs from microalgal biomass grown on piggery wastewater in a thin-layer cascade photobioreactor, identifying the critical environmental points of the proposed base process. The functional unit (FU) used in this

study was 1 m^3 of microalgae biomass with 95% of humidity, on which the results of the environmental impacts will be based on.

2.3.2. Life cycle inventory (LCI)

Life cycle inventory (LCI) consist of a quantification of the most important process inputs and outputs, within the system boundary (Ubando et al., 2022). In Figure 1, the system boundaries of the processes are illustrated, including energy, raw material, and obtained products while in Table 3 the LCI of the proposed process is displayed. Inventory data was determined in function of the material balance and the energy requirements, although the material used for equipment construction was not considered in the inventory analysis, since its contribution could be considered negligible (Golberg et al., 2021).

UAEE	**Unit**	**Value**
Enzymes	kg/FU	0.50
Electricity	kWh/FU	195.17
Cooling water	kg/FU	23.87
Solid separation	**Unit**	**Value**
Electricity	kWh/FU	8.14
Peptide purification	**Unit**	**Value**
Electricity	kWh/FU	0.33
Avoided peptide	kg/FU	5.13
Acid hydrolysis	**Unit**	**Value**
HCl	kg/FU	137.71
Water	m^3/FU	0.10
Electricity	kWh/FU	4.65
Low pressure steam	kWh/FU	66.19
Avoided char	kg/FU	8.68
PHA production	**Unit**	**Value**
Water	m^3/FU	3.88
NaOH	kg/FU	37.97

Electricity	kWh/FU	75.58
Low pressure steam	kWh/FU	94.27
Residual waste	m^3/FU	4.50
PHA purification	**Unit**	**Value**
NaCl	kg/FU	2.01
NaOH	kg/FU	1.00
C_2H_6O	kg/FU	39.60
Electricity	kWh/FU	2.83
Low pressure steam	kWh/FU	4.39
Avoided PHA	kg/FU	4.64
Residual waste	m^3/FU	0.09

Table 3. Life cycle inventory of the proposed process (FU = 1 m^3 of microalgae biomass).

The produced PHAs, peptides, and biochar, can be substitutes of similar compounds, avoiding their production emissions. For the first compound, the produced PHA was assumed to substitute polyethylene terephthalate (PET) according to a substitution rate PHA:PET equal to 1:0.72 (Asunis et al., 2021). On the other hand, peptides and biochar were considered as substitutes with ratio 1:1 of commercial proteins (Röder et al., 2022) and charcoal (Salimbeni, 2015).

2.3.3. Economic allocation

An economic allocation approach was used to divide the environmental impact between the different products of the process (Hermansson et al., 2020). The distribution, based on the economic value of the products, was calculated using the following equation 4 (Ardente & Cellura, 2012):

$$P_i = \frac{n_i \cdot x_i}{\sum_i n_i \cdot x_i} \qquad \text{Equation 4}$$

where P_i is the partitioning factor of the i (product), n_i is the quantity of the i (product), and x_i is the price of the i (product). These products correspond to peptides, PHAs and biochar produced in the biorefinery proposed process.

2.3.4. Life cycle impact analysis

The impact assessment method used in this study to determine the potential environmental impacts was the ReCiPe 2016 Midpoint (H) V1.08 / world (2010) H, considering 18 different categories, i.e. global warming (GW, expressed in kg CO_2 eq to air), stratospheric ozone depletion (SOD, expressed in kg trichlorofluoromethane (CFC_{11}) eq to air), ionizing radiation (IR, expressed in kBq Cobalt (Co_{60}) eq to air), ozone formation for human health (OZH, expressed in kg NO_x eq to air), fine particulate matter formation (FPM, expressed in kg $PM_{2.5}$ eq to air), ozone formation in ecosystems (OZE, expressed in kg NO_x eq to air), terrestrial acidification (TA, expressed in kg SO_2 eq to air), freshwater eutrophication (FE, expressed in kg P eq to freshwater), marine eutrophication (ME, expressed in kg N eq to freshwater), terrestrial ecotoxicity (TE, expressed in kg 1,4-dichlorobenzene (1.4-DCB) eq to industrial soil), freshwater ecotoxicity (FE, expressed in 1.4-DCB eq to freshwater), marine ecotoxicity (MEC, expressed in kg 1.4-DCB eq to marine water), human carcinogenic toxicity (HCT, expressed in kg 1.4-DCB eq to urban air), human non-carcinogenic toxicity (HNT, expressed in kg 1.4-DCB eq to urban air), land use (LU, expressed in m^2·year of crop land eq), mineral resource scarcity (MRS, expressed in kg Cu eq), fossil resource scarcity (FRS, expressed in kg oil eq consumed) and water consumption (WC, expressed in m^3 water eq consumed). This method provides a harmonised implementation of cause-effect pathways for the calculation of both midpoint and endpoint characterisation factors (Huijbregts et al., 2017). Also, it is a highly recommended method to obtain a first view on all environmental impacts by a new emerging green technology (as the microalgal biorefinery) (Thomassen et al., 2019), The software SimaPro^R 9.5 was employed to perform all the analysis, using the Ecoinvent 3.9.1 database.

3. RESULTS AND DISCUSSION

3.1. Techno-economic assessment (TEA)

3.1.1. Investment and operational costs

The proposed biorefinery base process involves an initial investment cost (IC) corresponding to the purchase of equipment and start-up of the plant, as well as annual operating costs related to raw materials and energy needs (OMC). Figure 2 illustrates the costs associated with each of the process sub-units showed in the Figure 1 in terms

of TEC (€), raw material (€/year), and operational costs (€/year) for process energy (electricity and heating) requirement. Regarding the TEC, all the process sub-units contributed similar percentage to the TEC, reaching a total of 1,654,189 €, with 23% contribution to PHAs production, 21% to solid separation, 20% to PHAs purification, 19% to acid hydrolysis, 13% to peptide purification and finally, only 3% to UAEE. With the TEC value obtained and applying the Lang's factors method mentioned above (section 2.2.1.), a total IC of 4,863,317 € was required for the proposed base process. As can be seen, the highest TEC (and thus, the highest IC) corresponded to the PHAs production line (including acid hydrolysis, PHAs production and PHAs purification sub-units) with a contribution of 62% (1,033,418 €) due to the greater amount of equipment required in it including centrifuges, pumps, and reactors. Therefore, this would be a hotspot in the base process to be optimized and reduced investment costs.

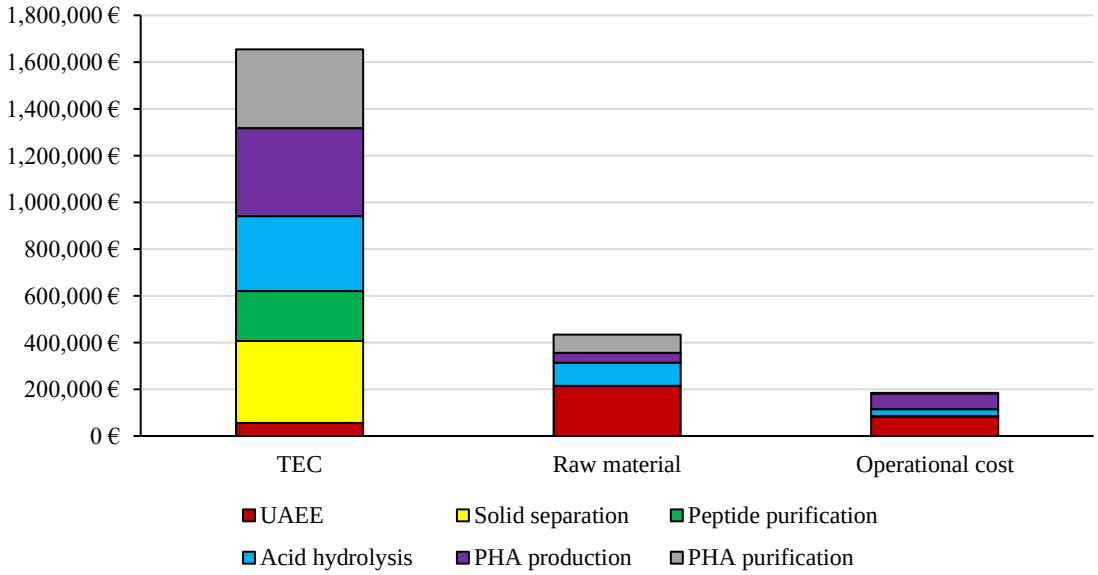
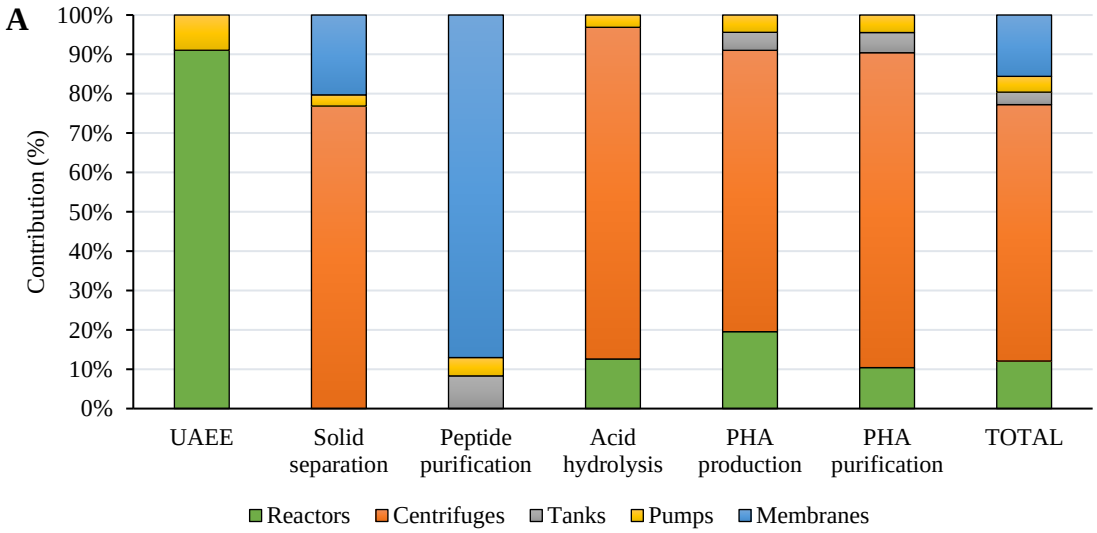


Figure 2. Total equipment cost (€), raw material and operational/ maintenance costs related to energy requirements (€/year) of the proposed biorefinery base process.

In each sub-unit of the process, the TEC depends on the type of equipment required as shown in Figure 3.A. It is noteworthy that in the sub-units of solid separation, acid hydrolysis, PHAs production and PHAs purification, centrifuges were the equipment that contribute most to the total cost (77%, 84%, 71% and 80% respectively). This resulted in an overall investment cost of 1.077.796 € only for this type of equipment which corresponds to 65% of the total TEC. This confirmed that the principal hotspot in the TEC was the high cost of centrifuges in the whole base biorefinery process. As a batch process, a centrifuge could be used for the peptide line

and another one for the PHAs line which allows to reduce the TEC in this type of equipment by 50%. Likewise, centrifuges could also be replaced by other separation technologies to reduce the IC. The use of membranes for solids separation could be a good alternative due to lower investment and operating costs (Zhao et al., 2023), but additional research would be necessary to evaluate the effect of the use of membranes in the mass and energy balances of this alternative technology. Kachrimanidou et al. (2021) designed a biorefinery process to produce 2.5 kT/year of poly(3-hydroxybutyrate) (PHB), crude phenolic extracts (CPE) and protein isolate (PI) in a biodiesel industry. The protein isolate production section had the highest contribution to the whole IC with 38% due to the high cost of centrifuges, as in this work. However, Andreola et al. (2023) produced protein hydrolyzates from mollusk and seafood wastes by enzymatic hydrolysis where the pretreatment (shredding and mincing) of the raw material was the largest contributor to the IC with almost 50%. These differences demonstrate the need for a techno-economic evaluation and optimization of each specific biorefinery process (Rojo et al., 2023a). In addition to centrifuges, the following types of equipment contributed the most to the total TEC: membranes (mainly in the peptide purification sub-unit with 186,637 €) with a contribution of 16% to the total TEC and reactors (mainly in the UAEE sub-unit with 50,791 €) with a contribution of 12% to the total TEC. Pumps (13) and storage tanks (3) only provided a contribution of less than 5% to the total TEC since as they were the cheapest equipment.



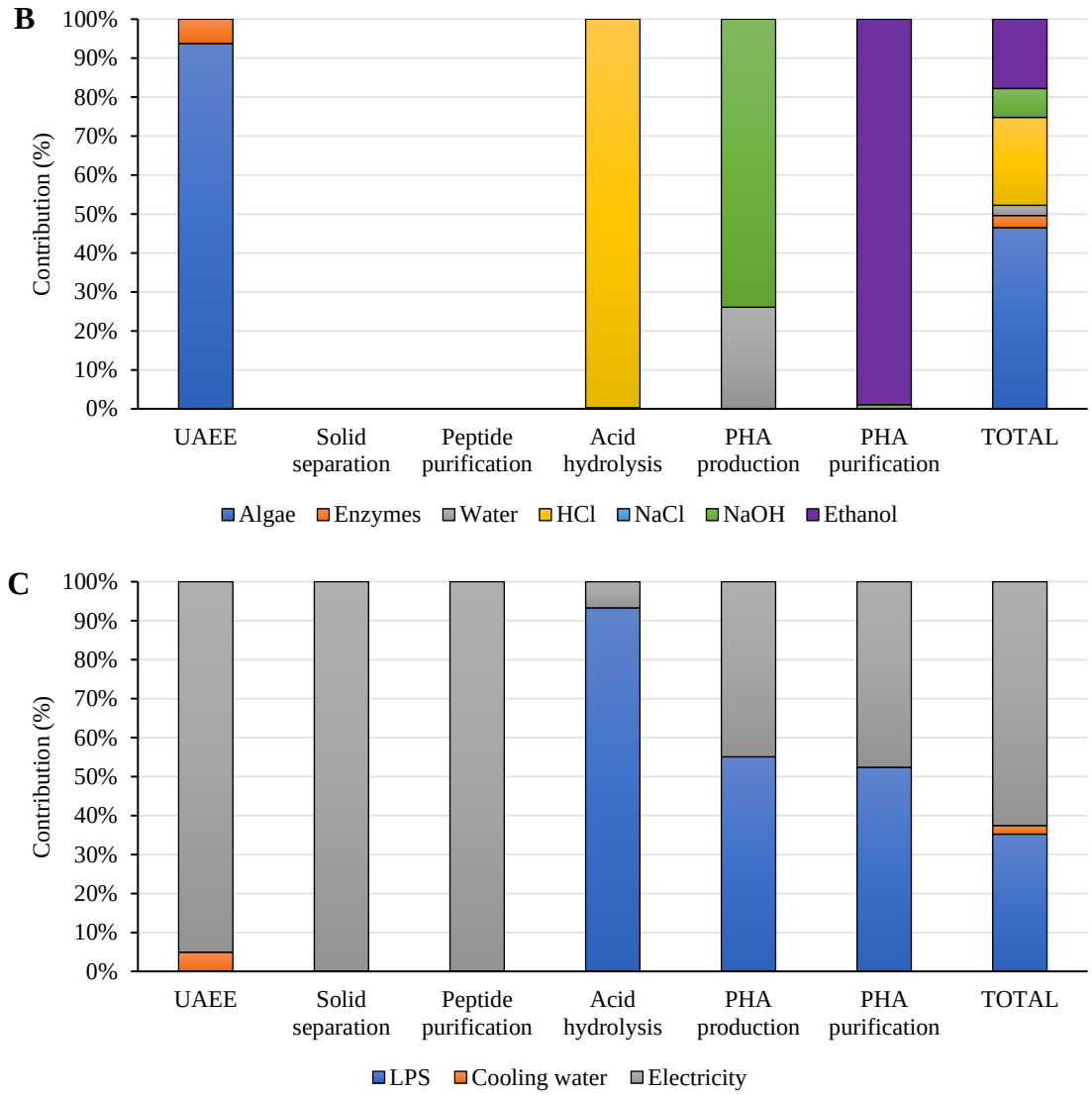


Figure 3. A) Total equipment cost (%), B) raw material (%) and C) operational costs (%) distribution in each process sub-unit of the proposed biorefinery base process.

Annual costs associated with the raw material and operational requirements of the proposed process were much lower than the TEC reaching a total of 434,166 €/year and 184,752 €/year respectively (Figure 2). Within the raw material, the main contributor is the UAEE sub-unit (50% and 215,200 €/year) due to the elevated annual cost of the microalgal biomass used as feedstock with an established price of 1.5 €/kg_{DCW}. On the other hand, the acid hydrolysis and PHAs purification sub-units contributed 23% (98,175 €/year) and 18% (78,446 €/year) respectively due to the requirements of HCl for the acid hydrolysis and ethanol for the solvent extraction process of PHAs. Finally, the PHAs production sub-unit contributed 10% (42,345 €/year) due to the requirements of NaOH for the acid hydrolyzate neutralization. Figure 3.B illustrates all these results, showing that algal biomass contributed 46% to the total raw material requirements of

the process, HCl contributed 23%, ethanol contributed 18% and NaOH contributed 7%, while the other raw materials (enzymes, water and, NaCl) contributed less than 4% to the total raw material requirement. From these results, the principal weakness in terms of raw material of the base biorefinery process was the high cost of the microalgal biomass as feedstock. Thus, it is still necessary to optimize the cultivation process in photobioreactors to reduce the production cost of this biomass and, therefore, the global cost of the process. Acién Fernández et al. (2019) pointed out that the technology could be improved to reduce prices up to 0.77 €/kg_{CDW} by maximizing the biomass productivity, reducing the cost of the reactors/technology and minimizing the manpower required for the operation of the facility.

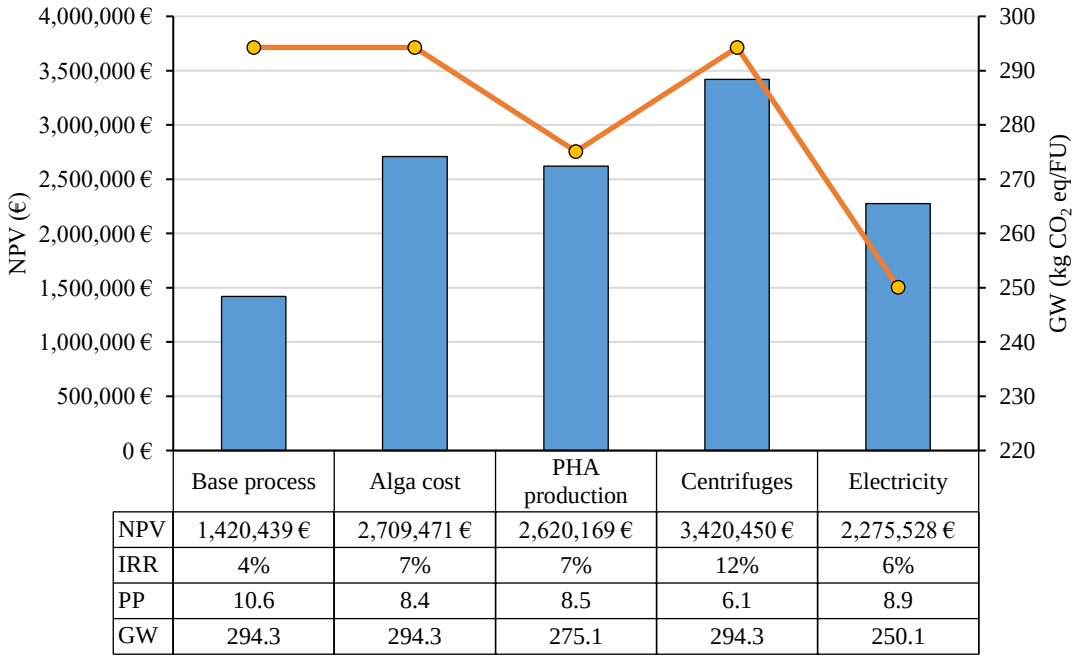
Finally, 3 sub-blocks contribute 97% of annual operation production costs, UAEE with 45%, PHAs production with 37% and acid hydrolysis with 15% (Figure 2). In these sub-blocks the main hotspots that should be improved were electricity and heating. The energy requirements for US operation led to a high electrical cost as shown in Figure 3.C with a 95% contribution within the UAEE sub-block (total of 78,458 €/year). Ultrasonication is a technology with high energy consumption (Amorim et al., 2020) but the combination with other technologies such as enzymatic allows for increased extraction yields and shorted extraction times (Rojo et al., 2023b), improving process sustainability despite moderate/high energy costs. As indicated by Rojo et al. (2023b), the combination of ultrasonication and enzymes allowed to reduce the hydrolysis time from 3 to 1 hour, increasing the solubilization and recovery yields. However, the high electricity consumption in our base process should be further reduced. Additional research should be addressed to optimize this step, reducing costs while achieving similar extraction yields. On the other hand, the high contribution of the PHA production sub-unit was related to the large heating requirements of the large-volume PHA reactor which results in a cost of 37,527 €/year for heating (55% of contribution within the sub-unit, Figure 3.C). Therefore, future research could be focused on decreasing the residence time of the reactor and, consequently, its volume. However, a proper integration of the heating flows in the plant could reduce or even cancel the reactor heating cost (pinch analysis). The high temperature necessary for microalgae biomass acid hydrolysis (120ºC) requires a high amount of steam with a heating cost of 26,346 €/year and a contribution of 93% within this sub-unit. This expense is necessary because previous research has shown that the solubilization yields

of acid hydrolysis of microalgae biomass decrease remarkably at temperatures lower than 120ºC (Rojo et al., 2023b), but residual heat of this stage can be used to heat the PHA reactor.

3.1.2. Technoeconomic assessment (TEA)

The economic feasibility of the proposed biorefinery base process was determined with the parameters defined in section 2.2. and the products prices established in Table 1 obtained a NPV, IRR, and PP of 1,42,439 €, 4%, and 10.6 years respectively. Since NPV is higher than 0, the proposed biorefinery process was economically feasible (Orive et al., 2021) and the investment was recovered around the year 10 of the total lifetime of the biorefinery plant. According to Romero-García et al. (2022), a project would be attractive for investors if the recovery of the investment occurs around 60–70% of the life of the project and in our case, this occurred in the 70% of the life project, so it was a feasible biorefinery process although there was further potential for economic improvement considering the founded weakness of the base process.

As shown in Figure 4 and considering the hotspots of the above section, if the microalgal biomass price was set at 0.77 €/kg_{DCW} (with the best cultivation conditions), an increasing in the NPV by 91% was observed, also achieving an IRR of 7% and a PP of 8.4 years. However, at present, it is very difficult to reach this production cost of the biomass and cultivation should be coupled with other processes such as CO_2 capture from flue gases of biogas upgrading (Das et al., 2022) along with the wastewater treatment. On the other hand, decreasing the number of centrifuges purchased (from 4 to 2) using each centrifuge in two different steps, could increase the NPV by 141% to reach 3,420,450 €, with an IRR and PP of 12% and 6.1 years respectively, although a correct integration of the operation times would be necessary. Other hotspot was the electricity requirement for UAEE sub-unit, but an increase in NPV of 60% could be achieved by reducing the electrical consumption of the ultrasounds system (considering only 10 min of pretreatment and a posterior enzymatic hydrolysis of 3 hours), with an IRR of 6% and a PP of 8.9 years. Finally, the low PHAs content of the microorganisms also influences on the base process, but by optimizing the operational conditions for PHA production or using genetic engineered microorganisms, it is possible to achieve remarkably the PHAs content of cells (Favaro et al., 2019; Mothes et al., 2007). If we increase this content percentage in our PHAs production sub-unit to 70%, the NPV would increase by 84% (IRR = 7% and PP = 8.5 years).



	Base process	Alga cost	PHA production	Centrifuges	Electricity
NPV	1,420,439 €	2,709,471 €	2,620,169 €	3,420,450 €	2,275,528 €
IRR	4%	7%	7%	12%	6%
PP	10.6	8.4	8.5	6.1	8.9
GW	294.3	294.3	275.1	294.3	250.1

Figure 4. Net present value (NPV), payback period (PP), internal rate of return (IRR) and global warming impact (GW) improvements of the design base process in the main critical points. Algal cost (reducing microalgal biomass cost from 1.5 €/kg_{DCW} to 0.77 €/kg_{DCW}), PHA production (increasing PHA content in microorganisms from 30% to 70%), centrifuges (reducing the number of centrifuges from 4 to 2) and electricity (reducing US electricity consumption to 10 min).

All this allowed us to conclude that the proposed base process is economically feasible thanks to the biorefinery concept of multi-compound production where all streams were utilized (Moldes et al., 2022). Nevertheless, two waste streams of the proposed process were not (liquid waste of the culture medium for PHAs production sub-unit and solvent waste from the PHAs purification sub-unit), since there is not enough scientific data about the chemical composition (as example, the waste from the PHAs production sub-unit it is possible to content sodium chloride and other compounds not used by the microorganisms) and the effect of using them in other process sub-units. So, although we achieved an economically sustainable process, this can be even better in a future work, improving the economic viability by optimizing the different hotspots of the proposed biorefinery process. In order to achieve this, future research should focus on the study of these critical points of the biorefinery process.

Nevertheless, all these economic indicators were determined with fixed selling prices (Table 2), and it was also possible to determine what would be the minimum selling price of the products, which prices were more volatile and dependent on the composition (peptides), considering a NPV equal to 0 (Ladakis et al., 2022). Fixing the

price of PHA, the minimum selling price of the peptide hydrolyzate stream would be 1.82 (€/kg), 9% lower than the price previously fixed for this product. Ladakis et al. (2022) proposed a biorefinery development using organic fraction from municipal solid waste with protein prices between 1 – 1.5 $/kg obtained by alkaline hydrolysis, while Andreola et al. (2023) designed a biorefinery process to produce protein hydrolyzates by enzymatic hydrolysis similar to our study from mollusc and fish residual, considering a selling price of 2 €/kg which can even be higher (up to 4 €/kg). If we consider the latter price for protein hydrolyzate, NPV, IRR, and PP of 17,247,238 €, 36%, 2.5 years were obtained, so it can be seen how the price of this product was the most influential in the economic sustainability of the biorefinery process, compared to PHAs price. However, it would also be necessary to increase the peptide concentration and its quality to be able to sell the product at this high price.

3.2. Life cycle assessment (LCA)

The environmental impact of the proposed base process was estimated using the LCI presented in Table 3. As shown in Table 4, where the results were presented based on the FU = 1 m^3 of microalgae biomass with 95% of humidity, several impact categories had moderate results, including GW (294.3 kg CO_2 eq/FU), IR (90.0 kBq Co_{60} eq/FU), TEC (1,214.4 kg 1.4-DCB eq/FU), HNC (296.8 kg 1.4-DCB eq/FU) and FRS (109.8 kg oil eq/FU). Focus on the GW category, the sub-unit contributing most to the total environmental impacts was PHAs production with 38% followed by acid hydrolysis with 29% and UAEE with 21%. On the other hand, UAEE sub-unit was the most contributor in the IR category (48%), followed by the PHAs production sub-unit (25%) and acid hydrolysis (25%). The high impact of the UAEE within GW and IR categories was due to the high electricity needs of ultrasonication which contributed with 53.1 kg CO_2 eq in the GW category and 40.9 kBq Co_{60} eq in the IR category. So, the high electricity requirement was a weakness in the environmental assessment of the base biorefinery process which must be reduced as well as indicated in the technoeconomic evaluation to diminish operation costs. The electricity was also the highest contributor to global warming potential (GWP) with 86% in the biorefinery process designed by Sreekumar et al. (2020) to produce ethanol from rice straw by enzymatic hydrolysis and in the biorefinery process proposed by López-Herrada et al. (2023) to produce microalgae-based fungicide with a consumption of 1.4 kWh/kg biomass. This impact depends on the origin of electricity production, and, in Spain, the

energy produced come mainly from non-renewable sources such as coal, fuel/gas with percentages varying between 57.2% and 78.4% (López-Herrada et al., 2023). This explains the high impact related to electricity, which according to the database of Ecoinvent (electricity mix of Spain) produced 0.27 kg CO_2 eq/kWh. The use of renewable energy for electricity production could reduce this impact (Sreekumar et al., 2020). However, by making the same improvement as in the TEA (reducing the US treatment to 10 min followed by an enzymatic hydrolysis of 3 hours), the GW impact could be reduced by 15% up to 250.1 kg CO_2 eq/FU, showing the considerable impact of this energy due to its fossil origin.

Likewise, PHAs production had a moderate contribution to the GW and IR impact categories, related to heating (40% in GW category) and electricity (37% in GW category and 88% in IR category). In this sub-unit, increasing the PHAs content in microorganism would improve the economic sustainability of the process (section 3.1), but also the environmental impact as more PHAs would be produced which was considered a substitute of PET (and thus, an avoided emission) in the life cycle inventory, reducing the global warming impact by 7% (275.1 kg CO_2 eq).

Due to the wide variety of biorefinery processes that can be designed and the different methods for life cycle assessment (functional unit, LCIA, allocation method…), comparison with another research was complicated and is not very feasible in this type of analysis (Saravanan et al., 2023). However, it was possible to compare with other types of petroleum-based plastics included in the Ecoinvent database, such as polyethylene terephthalate (PET), polyethylene (PE), polypropylene (PP) or polystyrene (PS) which can be substituted by PHAs. From the Ecoinvent database, we obtained a GW impact of 3.1, 2.4, 2.4 and 3.9 kg CO_2 eq/kg for each respectively. In our study, and after applying the economic allocation, the obtained GW impact was 3.4 kg CO_2 eq/kg PHA, a value greater than all the above plastics (except for PS). Thus, it is necessary to improve the proposed process with the above developments to enhance the environmental behavior, such as the electricity requirement for US operation which could allow to reduce the GW impact up to 2.9 kg CO_2 eq /kg PHA.

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Category	Unit/FU	UAEE	Solid separation	Protein purification	Acid hydrolysis	PHA production	PHA purification	Total
GW	kg CO_2 eq	6.12×10^{1}	2.22×10^{0}	-6.75×10^{0}	8.49×10^{1}	1.12×10^{2}	4.04×10^{1}	***2.94×10^{2}***
SOD	kg CFC_{11} eq	2.89×10^{-5}	1.09×10^{-6}	-5.58×10^{-5}	5.81×10^{-5}	7.39×10^{-5}	-8.54×10^{-5}	***2.08×10^{-5}***
IR	kBq Co-60 eq	4.35×10^{1}	1.71×10^{0}	-7.30×10^{-1}	2.24×10^{1}	2.27×10^{1}	4.96×10^{-1}	***9.00×10^{1}***
OFH	kg NO_x eq	1.46×10^{-1}	5.37×10^{-3}	-3.31×10^{-2}	1.80×10^{-1}	2.39×10^{-1}	9.24×10^{-2}	***6.30×10^{-1}***
FPM	kg $PM_{2.5}$ eq	8.26×10^{-2}	2.93×10^{-3}	-1.14×10^{-2}	1.83×10^{-1}	1.61×10^{-1}	3.35×10^{-2}	***4.52×10^{-1}***
OFT	kg NO_x eq	1.53×10^{-1}	5.61×10^{-3}	-3.42×10^{-2}	1.85×10^{-1}	2.49×10^{-1}	1.09×10^{-1}	***6.67×10^{-1}***
TAC	kg SO_2 eq	2.06×10^{-1}	7.47×10^{-3}	-2.45×10^{-2}	5.26×10^{-1}	3.42×10^{-1}	9.75×10^{-2}	***1.15×10^{0}***
FEU	kg P eq	1.56×10^{-2}	4.40×10^{-4}	-3.34×10^{-3}	4.92×10^{-2}	4.41×10^{-2}	1.80×10^{-2}	***1.24×10^{-1}***
MEU	kg N eq	2.07×10^{-3}	6.62×10^{-5}	-6.17×10^{-2}	6.92×10^{-3}	2.41×10^{-2}	5.99×10^{-4}	***-2.79×10^{-2}***
TEC	kg 1.4-DCB	1.28×10^{2}	4.28×10^{0}	-5.62×10^{1}	7.04×10^{2}	3.36×10^{2}	9.80×10^{1}	***1.21×10^{3}***
FEC	kg 1.4-DCB	2.02×10^{0}	6.82×10^{-2}	-1.09×10^{0}	7.58×10^{0}	3.74×10^{0}	1.16×10^{-1}	***1.35×10^{1}***
MEC	kg 1.4-DCB	2.61×10^{0}	8.70×10^{-2}	-1.38×10^{0}	9.95×10^{0}	4.94×10^{0}	1.52×10^{0}	***1.77×10^{1}***
HCT	kg 1.4-DCB	8.44×10^{0}	9.36×10^{-2}	-5.71×10^{-1}	8.40×10^{0}	5.32×10^{0}	1.30×10^{0}	***2.30×10^{1}***
HNC	kg 1.4-DCB	4.01×10^{1}	1.30×10^{0}	-1.05×10^{1}	1.51×10^{2}	9.54×10^{1}	2.00×10^{1}	***2.97×10^{2}***
LU	m^2a crop eq	1.49×10^{0}	5.38×10^{-2}	-5.30×10^{1}	-6.80×10^{0}	2.17×10^{0}	4.63×10^{-1}	***-5.56×10^{1}***
MRS	kg Cu eq	2.15×10^{-1}	5.29×10^{-3}	-4.77×10^{-2}	5.50×10^{-1}	2.70×10^{-1}	8.01×10^{-2}	***1.07×10^{0}***
FRS	kg oil eq	1.76×10^{1}	6.49×10^{-1}	-1.70×10^{0}	3.02×10^{1}	3.07×10^{1}	3.23×10^{0}	***1.10×10^{2}***
WC	m^3	2.40×10^{1}	2.10×10^{-2}	-2.23×10^{1}	2.35×10^{0}	-9.42×10^{-2}	1.71×10^{-1}	***4.17×10^{0}***

Table 4. Environmental impact results of proposed biorefinery process by ReCiPe 2016 Midpoint (H) V1.08 / world (2010) H.

4. CONCLUSIONS

TEA and LCA are two important tools to analyse the feasibility of different processes, which could identify the hotspots of the designed system. The production of peptides and PHA from microalgae biomass grown on piggery wastewater was a feasible process with a NPV of 1,420,439 € and a global warming of 294.3 kg CO_2 eq/FU, although the TEA identified various hotspots. These including the high cost of centrifuges, the high production cost of the biomass produced in open photobioreactors fed with wastewater, low PHAs production by microorganism and, the high electricity requirement for ultrasonication. Likewise, the LCA founded that electricity contributed the most to the global warming category due to the high necessity and the fossil origin of electricity production in Spain. Thus, these limitations should be studied and optimised to achieve a more sustainable biorefinery process, with a possible global warming reduction of 15% (by optimizing the electricity requirement of ultrasonication extraction) and/or NPV improvement of up to 141%, 91% and, 84% (by reducing the number of centrifuges, the microalgal biomass cost and by enhancing PHAs content in microorganism respectively).

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CONCLUSIONES Y TRABAJO FUTURO

El uso de aguas residuales como fuente de nutrientes en la biorrefinería de microalgas permite reducir los costes de producción de la biomasa microalgal, así como obtener agua útil para el riego agrícola y una valiosa materia prima que puede ser empleada en la producción sostenible de diferentes bioproductos.

La biomasa algal puede ser utilizada para aplicaciones agrícolas como bioestimulante y/o biopesticida. Se estudiaron varios escenarios en la producción de estos dos bioproductos a partir de la valorización de biomasa algal cultivada en aguas residuales de purines (37,8 m^3/d procedentes de dos granjas porcinas del municipio de Cuéllar) comparando el uso de centrífuga y membranas como métodos de recolección. Analizando el uso de la biomasa algal completa como bioestimulante, la centrifugación resultó en un coste de producción de bioestimulante de 342,6 €/m^3 con elevados costes de inversión (1.911.553 €) y operación, los cuales se redujeron (1.042.401 € de coste de inversión) al utilizar un sistema de membranas hasta 65,5 €/m^3, aunque el producto obtenido era ~4,5 veces menos concentrado. Así mismo, el uso de membranas para el cosechado de biomasa fue más sostenible medioambientalmente que la centrifugación consiguiendo una huella de carbono de 217 kg CO_2/ha de cultivo (mediante el uso de un sistema de captura in situ de CO_2 de gases de combustión con separación por membranas). En ambas evaluaciones (análisis económico y medioambiental), el transporte final del bioestimulante producido tuvo un gran impacto en el coste y la huella de carbono, siendo el principal punto crítico del sistema por lo que la planta debería construirse cerca de cultivos agrícolas.

Por otro lado, la combinación de producción de biopesticidas y bioestimulantes incrementó la inversión (hasta 3.494.069 € utilizando centrífuga) y los costes de operación debido a la mayor complejidad del proceso de biorrefinería, que sufrió una influencia significativa con los cambios del 10% en los precios de la electricidad y calentamiento (hasta un 18% de variación). Sin embargo, no existe suficiente información experimental sobre la producción de biopesticidas para calcular el impacto ambiental de este proceso.

La biomasa algal cultivada en fotobiorreactores de tratamiento de aguas residuales ha demostrado ser una materia prima sostenible para aplicaciones agrícolas, pero la recuperación fraccionada de los componentes acumulados en las células algales

es otra alternativa prometedora. Las proteínas y los carbohidratos son los principales componentes de esta biomasa, y pueden extraerse mediante diferentes métodos.

La hidrólisis enzimática es un tratamiento suave con bajos rendimientos de solubilización. El análisis ANOVA de un diseño experimental de hidrólisis enzimática mostró el efecto significativo de tres parámetros estudiados: el tipo de enzima (Alcalasa, Protamex y Celluclast), el tiempo de hidrólisis (1, 3 y 5 horas) y el tipo de biomasa algal (consorcio de microalgas y bacterias y microalgas puras) sobre los rendimientos de solubilización y recuperación. La solubilización de proteínas fue mayor en el caso de las microalgas pura, especialmente después de utilizar la enzima Alcalasa, aumentando del 38.9% después de 1 hora al 64.9% después de 5 horas. Por lo tanto, se observó un claro efecto del tiempo de hidrólisis sobre la solubilización de proteínas utilizando esta enzima. La Alcalasa también proporcionó mayores rendimientos de solubilización que la enzima Protamex (<40%) y, como era de esperar, Celluclast proporcionó los rendimientos más bajos de solubilización de proteínas. Según el análisis ANOVA, todos los parámetros estudiados tuvieron un efecto significativo en la solubilización de proteínas, siendo de nuevo el tipo de enzima el más importante (con una contribución del 59.6%). Para el consorcio de microalgas y bacterias, se alcanzaron rendimientos de recuperación de péptidos del 32.4%, 19.6% y 6.6% para Alcalasa, Protamex y Celluclast, mientras que para la biomasa microalgal pura, la recuperación de péptidos fue del 34.2%, 13.7% y 5.3% respectivamente. Sin embargo, aunque la enzima Alcalasa permitió la recuperación de más péptidos que otras enzimas, Protamex dio lugar a péptidos de mayor tamaño que la Alcalasa, obteniéndose cuatro bandas con peso moleculares de 135, 75, 63 y 11 kDa.

Por otro lado, los mayores rendimientos de solubilización y recuperación de carbohidratos (38.5%) se alcanzaron con la enzima Celluclast, el consorcio de microalgas y bacterias y un tiempo de hidrólisis de 5 horas, resultando el tipo de enzima el parámetro más importante. Este fue el único experimento en el que no se observaron pérdidas de carbohidratos, pero en promedio se obtuvieron pérdidas ligeramente superiores en la biomasa pura (13.7%) que en el consorcio (12.1%). Así, se obtuvieron menores recuperaciones de monosacáridos en las microalgas puras (3.7 – 21.1%) que en la biomasa microalga-bacteria (20.5 - 34%) con notables pérdidas de xilosa en ambos casos. La microscopía electrónica también reveló un alto grado de rugosidad de la pared celular en la biomasa de microalgas-bacterias que en las microalgas puras.

La presencia de bacterias modificó la estructura de la pared celular de la biomasa e influyó en la posterior valorización por hidrólisis enzimática, que es un método muy dependiente de la estructura y composición de la biomasa. Las imágenes microscópicas no mostraron ninguna ruptura de la pared celular, lo que indicó que los péptidos y monosacáridos extraídos formaban parte de la pared celular y, por tanto, sería posible mejorar la eficacia de la extracción. Así, se realizaron nuevos estudios para abordar la aplicación de otros tratamientos de disrupción celular para aumentar la extracción de proteínas y carbohidratos, pero siempre utilizando condiciones suaves.

Con el fin de aumentar los rendimientos de la hidrólisis enzimática, se aplicaron por primera vez nuevos métodos de extracción a la biomasa cultivada en fotobiorreactores combinando ultrasonidos y microondas con hidrólisis enzimática con Protamex (UAEE y MAEE). Los resultados de estos nuevos métodos se compararon con los tratamientos químicos (hidrólisis alcalina y ácida con NaOH 2M y HCl 2M respectivamente) y comúnmente utilizados para la ruptura de la pared celular a diferentes temperaturas (40, 60 y 120ºC). La hidrólisis alcalina a 120ºC alcanzó los mayores rendimientos de solubilización de proteínas (90%) y recuperación de péptidos (81%), con un notable efecto de la temperatura y tamaños de péptidos muy pequeños. La disminución de la temperatura de hidrólisis alcalina disminuyó los rendimientos de solubilización, pero no la degradación de las proteínas. Por otro lado, la hidrólisis ácida a 120ºC solubilizó el 75% de las proteínas iniciales, disminuyendo al 19% a 40 y 60ºC. La hidrólisis ácida produjo una elevada degradación de las proteínas en otros compuestos, sin presencia de grandes péptidos en los hidrolizados (no se detectaron bandas por electroforesis). Por el contrario, la UAEE proporcionó recuperaciones moderadas de péptidos (39 - 44%, superiores a los controles de hidrólisis enzimática), y los mejores resultados en términos de pureza (46 - 47%), tamaño (hasta 135 kDa) y contenido moderado de aminoácidos esenciales (33.3%) de los péptidos en comparación con los tratamientos químicos. Esta mejora se atribuyó a la cavitación producida por los ultrasonidos, que potenció la disrupción celular y mejoró el rendimiento de solubilización de la hidrólisis enzimática con bajas pérdidas de proteínas. Sin embargo, ni la aplicación de microondas ni la adición de Celluclast al Protamex mejoraron significativamente el rendimiento de extracción de ninguno de los experimentos de hidrólisis enzimática asistida.

En cuanto a la fracción de carbohidratos, la hidrólisis ácida a 120ºC consiguió la mayor solubilización de carbohidratos (86.8%) y recuperaciones de monosacáridos (69% de la glucosa inicial y 57% de la xilosa inicial) con bajas pérdidas a esta temperatura, lo que pudo estar relacionado con un efecto esterilizante. En contraste con las proteínas, el efecto de la temperatura fue menor en la solubilización de carbohidratos por tratamientos químicos mientras que la asistencia de microondas mejoró la solubilización de carbohidratos por la hidrólisis enzimática, especialmente en el caso de la xilosa (>70%) debido a la disrupción de los grupos acetilo de la hemicelulosa en la pared celular. MAEE con proteasa permitió obtener una solubilización final de carbohidratos del 57.9%. No se encontró recuperación de xilosa en ningún experimento enzimático (hidrólisis enzimática, UAEE y MAEE) y utilizando el cóctel Celluclast y Protamex apareció un nuevo pico en el análisis por HPLC de estos hidrolizados indicando una probable isomerización de la xilosa en xilulosa.

Por tanto, la combinación de ultrasonidos y enzimas permitió mejorar la eficiencia de la hidrólisis enzimática en condiciones suaves, aunque los rendimientos de solubilización y recuperación siguen siendo inferiores a los obtenidos con tratamientos químicos, pero con tamaños de péptidos elevados. Así pues, es posible recuperar proteínas y carbohidratos a partir de biomasa algal en fotobiorreactores de tratamiento de aguas residuales y la elección del tratamiento de extracción dependerá de la aplicación y de las características del producto final deseado (tamaños y concentración de péptidos, recuperación de monosacáridos como precursores de otros productos de alto valor añadido). No obstante, el uso de aguas residuales de purines como fuente de nutrientes para el cultivo de microalgas también conlleva la presencia de contaminantes emergentes como antibióticos veterinarios y/o metales que pueden afectar a su composición macromolecular, procesos de valorización y la aplicación final de los bioproductos.

El dopaje de la alimentación de purines de cerdo del fotobiorreactor con varios contaminantes (sulfadiazina, tetraciclina y ciprofloxacina a 100 µg/L como antibióticos veterinarios (VA) y cobre, zinc a 20 mg/L y arsénico a 30 µg/L como metales (HM)) provocó una disminución de la concentración de biomasa en el fotobiorreactor de hasta un 35%. La presencia de ambos tipos de contaminantes provocó un estrés oxidativo en el medio de cultivo, que aumentó el contenido proteico de la biomasa de microalgas (en un 30%) para superar el desequilibrio debido a la sobreproducción de especies reactivas

del oxígeno a expensas del contenido de glucosa (reducido en un 42%). Además, se modificó la estructura de la pared celular como mecanismo de defensa contra el estrés por antibióticos y metales para preservar las células de daños externos. Estos cambios estructurales y composicionales afectaron a los posteriores procesos de valorización llevados a cabo (hidrólisis química a 120ºC, hidrólisis enzimática con proteasa, ultrasonicación y UAEE con proteasa) debido a la influencia de la estructura de la biomasa en el rendimiento de la extracción.

Los rendimientos de solubilización y recuperación de proteínas aumentaron en presencia de VA y/o HM tras los tratamientos químicos, pero disminuyeron tras los tratamientos con ultrasonidos y enzimáticos. La solubilización de la glucosa se vio muy reducida por los VA y los HM en todos los tratamientos estudiados. El efecto de los contaminantes sobre la solubilización de la xilosa fue similar al de las proteínas, pero aumentó la degradación reduciendo su recuperación. Por último, se encontró un alto porcentaje de metales dopados en los hidrolizados de los tratamientos químicos en comparación con los métodos físicos y biológicos (<20%).

Todos estos métodos de extracción permiten obtener un hidrolizado con concentraciones moderadas/altas de péptidos y monosacáridos que puede utilizarse en la industria, ya que no es apto para usos humanos debido a la presencia de bacterias. El uso de la fracción proteica de la biomasa algal para aplicaciones industriales requiere la recuperación de péptidos con alto peso molecular y, por tanto, buenas propiedades tecno-funcionales. La combinación de ultrasonidos e hidrólisis enzimática proporcionó los mayores rendimientos de recuperación de péptidos de alto peso molecular, pero también un sólido residual rico en componentes valiosos. La hidrólisis ácida permite una solubilización prácticamente total de esta biomasa residual que incluye algunos aminoácidos, monosacáridos y diferentes subproductos de la degradación. Esta mezcla de componentes podría ser útil como medio de cultivo para microorganismos capaces de acumular polihidroxialcanoatos (PHAs) para la producción de bioplásticos. Sin embargo, como primer paso, es necesario estudiar la viabilidad económica y medioambiental de esta alternativa de biorrefinería mediante análisis tecno-económicos y de ciclo de vida (TEA y LCA), identificando los principales puntos críticos de esta idea de valorización fraccionada.

La producción de péptidos y PHAs a partir de biomasa algal cultivada con aguas residuales de purines es un proceso viable con un valor actual neto (VAN) de ~1.420.000 € tras un coste de inversión de ~4.863.000 €, y un impacto de huella de carbono (GW) de 294 kg CO_2 eq/m^3 de biomasa de microalgas (95% de humedad), aunque el TEA identificó varios puntos críticos. Entre ellos, el elevado coste de las centrífugas (65% del coste total del equipo), el elevado coste de producción de la biomasa producida en fotobiorreactores abiertos alimentados con aguas residuales, el bajo contenido en PHAs de los microorganismos seleccionados inicialmente y la elevada necesidad de electricidad para la ultrasonicación. Mejorando estos puntos conflictivos identificados, fue posible aumentar la sostenibilidad económica del proceso propuesto hasta un 141% (reduciendo el número de centrifugadoras), un 91% (reduciendo el coste de la biomasa hasta 0,77 €/kg_{CDW}) y un 84% (aumentando el contenido de PHAs en los microorganismos hasta un 70%).

Así mismo, el LCA encontró que la electricidad era el principal contribuyente al impacto sobre el calentamiento global (50%) debido al alto origen fósil de la producción de electricidad en España. Por lo tanto, esta limitación debe ser estudiada y optimizada para lograr un proceso de biorrefinería más sostenible en el futuro, con una reducción de GW de hasta 250 kg CO_2 eq/FU tras la optimización y reducción de los requisitos de electricidad de la extracción por ultrasonidos, que es el principal contribuyente a las necesidades de electricidad del proceso. Estos resultados mostraron la importancia de llevar a cabo un análisis tecno-económico y de ciclo de vida para identificar los puntos débiles de un proceso en una etapa temprana, ayudando a evitar altos costes económicos y ambientales antes de la implementación de una tecnología emergente como la biorrefinería de microalgas.

Basándose en todos estos resultados, la investigación futura podría centrarse en los siguientes puntos:

- Optimización de la etapa de extracción con disolventes de la producción de biopesticidas, análisis fisicoquímico del producto y estudios agronómicos y de laboratorio de la eficacia de los biopesticidas.
- Análisis del ciclo de vida de la producción de biopesticidas a partir de biomasa cultivada en aguas residuales de purines.

- Optimización de los parámetros de operación (tiempo de hidrólisis, energía, tipo de enzima, relación biomasa/enzima, temperatura...) de la UAEE.
- Estudio y desarrollo de un método analítico de análisis químico de antibióticos veterinarios en los hidrolizados obtenidos.
- Investigación y optimización de los principales puntos críticos del proceso de biorrefinería propuesto para la producción de péptidos y PHAs.
- Optimización de métodos para la separación de monosacáridos y péptidos de los hidrolizados y purificar los péptidos recuperados.
- Optimización de los métodos de recuperación y purificación de PHAs de los microorganismos.

CONCLUSIONS AND FUTURE WORK

The use of wastewater as source of nutrients in microalgae biorefinery leads to reduce production costs of microalgal biomass, obtaining clean water useful for agricultural irrigation and a valuable raw material which can be used for sustainable production of different bioproducts.

The algal biomass can be used for agriculture applications as biostimulant and/or biopesticide. Several scenarios were studied in the production of these two bioproducts from the valorization of algal biomass grown on piggery wastewater (37.8 m^3/d from two pig farms in the municipality of Cuéllar) comparing centrifugation and membranes as harvesting methods. Analyzing the use of whole algal biomass produced as biostimulant, centrifugation resulted in a biostimulant production cost of 342.6 €/m^3 with high investment (1,911,553 €) and operating costs which were reduced (1,042,401 € of investment cost) by using a membrane system up to 65.5 €/m^3, although the obtained product was 4.5 time less concentrated. Likewise, using membranes for biomass harvesting was more environmentally sustainable than centrifugation achieving a final footprint of 217 kg CO_2/ha crops (by using an on-site capture CO_2 system from flue gases with membrane separation). In both types of assessments (economic and environmental analysis), the final transport of the produced biostimulant had a great impact on the final cost and footprint, which was the main hotspot of the system so the plant should be built near agricultural crops.

On the other hand, the coupling of the production process of biopesticides with biostimulants increased the investment (up to 3,494,069 € using centrifuge) and operation costs due to the high complexity of the biorefinery process, which suffered a significant influence with the changes of 10% in the prices of electricity and heating (up to 18% of variation). However, there is not enough experimental information about the production of biopesticides to calculate the environmental impact of this coupled process.

The algal biomass grown on wastewater treatment photobioreactors has proven to be a sustainable raw material for agriculture applications, but the fractional recovery of the components accumulated in the cells is another promising alternative. Proteins and carbohydrates are the major components of this biomass, and they can be extracted by different methods.

Enzymatic hydrolysis is a mild treatment with low solubilization yields. The ANOVA analysis of an experimental design of enzymatic hydrolysis showed the significant effect of the three studied parameters: the type of enzyme (Alcalase, Protamex and Celluclast), the hydrolysis time (1, 3 and 5 hours) and type of algal biomass (consortium of microalgae and bacteria and pure microalgae) on the solubilization and recovery yields. Protein solubilization was higher for the pure microalgae, especially after using Alcalase enzyme, increasing from 38.9% after 1 hour to 64.9% after 5 hours. Thus, a clear effect of the hydrolysis time on protein solubilization was observed using this enzyme. Alcalase provided higher solubilization yields than Protamex enzyme (<40%) and, as expected, Celluclast provided the lowest protein solubilization yields. According to the ANOVA analysis, all the studied parameters had a significant effect on the protein solubilization, with the type of enzyme being again the most important one (contribution of 59.6%). For the bacterial and microalgal biomass, peptide recovery yields of 32.4%, 19.6%, and 6.6% for Alcalase, Protamex, and Celluclast were achieved, while for the pure microalgal biomass, peptide recovery was 34.2%, 13.7%, and 5.3% respectively. However, although Alcalase allowed for the recovery of more peptides than other enzymes, Protamex resulted in higher peptides sizes than Alcalase, obtaining four bands with molecular weights of 135, 75, 63 and about 11 kDa.

On the other hand, the highest carbohydrate solubilization and recovery yields (38.5%) were achieved with Celluclast, microalgae-bacteria consortium and a hydrolysis time of 5 hours, resulting the type of enzyme the most important parameter. This was the only experiment where no carbohydrate losses were observed, but on average slightly higher losses were obtained in the synthetic biomass (13.7%) than in the consortium biomass (12.1%). So, lower monosaccharide recoveries were obtained in the pure microalgae grown on synthetic media (3.7 – 21.1%) than in the microalgae-bacteria biomass (20.5 – 34%) with remarkable xylose losses in both cases. Electronic microscopy revealed high degree of cell wall roughness on microalgae-bacteria biomass than pure microalgae.

The presence of bacteria modified the cell wall structure of the biomass and influenced the posterior valorization by enzymatic hydrolysis, which is a method that is highly dependent on the structure and composition of the biomass. Microscopic images showed no disruption in the cell wall, which indicates that the extracted peptides and

monosaccharides were part of the cell wall and therefore it would be possible to improve the extraction efficiency. Thus, further studies were performed to address the application of other treatments for cell disruption to increase the protein and carbohydrate extraction, but always using mild conditions.

In order to increase the enzymatic hydrolysis yields, new extraction methods were applied for the first time to biomass grown on wastewater treatment plants by combining ultrasounds and microwaves with enzymatic hydrolysis with Protamex (UAEE and MAEE). The results of these new methods were compared to chemical treatments (alkaline and acid hydrolysis with NaOH 2M and HCl 2M respectively) and commonly used for cell wall breakthrough at different temperatures (40, 60 and 120ºC). Alkaline hydrolysis at 120ºC achieved the highest protein solubilization (90%) and peptide recovery (81%) yields, with notable effect of temperature and very small peptide sizes. The decrease in temperature of alkaline hydrolysis decreased the solubilization yields but not the degradation of proteins. On the other hand, acid hydrolysis at 120ºC solubilized 75% of the initial proteins, decreasing to 19% at 40 and 60ºC. Acid hydrolysis resulted in high degradation of proteins into other compounds with no presence of large peptides in the hydrolyzates (no detected bands by electrophoresis). On the contrary, the UAEE provided moderated peptide recoveries (39–44%, higher than enzymatic hydrolysis controls), and the best results in terms of purity (46–47%), size (up to 135 kDa) and moderate content of essential amino acids (33.3%) of the peptides compared to the chemical treatments. This improvement was attributed to the cavitation produced by ultrasounds which enhanced cellular disruption and improved the solubilization yield of the enzymatic hydrolysis with low protein losses. However, nor the application of microwaves and nor the addition of Celluclast to Protamex did not significantly improve the protein extraction yield of any of the assisted enzymatic hydrolysis experiments.

Concerning carbohydrate fraction, acid hydrolysis at 120ºC achieved the highest solubilization of carbohydrates (86.8%) and recoveries of monosaccharides (69% of initial glucose and 57% of initial xylose) with low losses at this temperature which could be related to a sterilizing effect. In contrast to the proteins, the effect of temperature was lower on the solubilization of carbohydrates by chemical treatments while microwaves assistance enhanced carbohydrate solubilization by the enzymatic hydrolysis, especially in the case of xylose (>70%) due to the disruption of the acetyl

groups of hemicellulose in the cell wall. MAEE with protease allowed to obtain a final carbohydrate solubilization of 57.9%. No recovery of xylose was found in any enzymatic experiment (enzymatic hydrolysis, UAEE, and MAEE). Using the Celluclast and Protamex cocktail a new peak appeared in HPLC analysis of these hydrolyzates indicating a probable xylose isomerization to xylulose.

Therefore, the combination of ultrasounds and enzymes allowed to improve the efficiency of enzymatic hydrolysis at mild conditions, although solubilization and recovery yields are still lower than with chemical treatments but with high peptide sizes. So, it is possible to recover proteins and carbohydrates from biomass grown on wastewater treatment photobioreactors and the selection of the extraction treatment will depend on the application and characteristics of the final desired product (peptide sizes and concentration, monosaccharide recovery as precursors for high-added value products). Nevertheless, the use of piggery wastewater as source of nutrients for microalgae cultivation also leads to the presence of emerging pollutants such as veterinary antibiotics and/or metals which affect its macromolecular composition, valorization processes and final application of bioproducts.

The doping of pig manure fed to the photobioreactor with the pollutants (sulfadiazine, tetracycline, and ciprofloxacin at 100 µg/L as veterinary antibiotics (VA) and copper, zinc at 20 mg/L and arsenic at 30 µg/L as metals (HM)) resulted in a diminution of the biomass concentration in photobioreactor by up to 35%. The presence of both type of contaminants caused oxidative stress in the culture media which. increased the protein content of microalgae biomass (by 30%) to overcome the imbalance due to overproduction of reactive oxygen species at the expense of the glucose content (reduced by 42%). Also, the cell wall structure was modified as a defence mechanism against antibiotic and metal stress to preserve algae cells from the external damage. These structural and compositional changes affected the posterior valorization processes undertaken (chemical hydrolysis at 120ºC, enzymatic hydrolysis with protease, ultrasonication and UAEE with protease) due to the influence of biomass structure on the extraction performance.

Protein solubilization and recovery yields increased in presence of VA and/or HM after chemical treatments but decreased after ultrasound and enzymatic treatments. Glucose solubilization was greatly reduced by VA and HM for all the studied

treatments. The effect of contaminants on xylose solubilization was similar than on proteins, but they increased degradation reducing its recovery. Finally, high percentage of doped metals was found in the hydrolyzates of chemical treatments compared to physical and biological methods (<20%).

All these extraction methods allow to obtain a hydrolyzate with moderate/high concentrations of peptides and monosaccharides which can be used in industry since it is not suitable for human uses due to the bacteria presence. The use of the protein fraction from algal biomass for industrial applications requires the recovery of peptides with high molecular weight, and therefore good techno-functional properties. The combination of ultrasounds and enzymatic hydrolysis provided the highest recovery yields of high molecular weight peptides, but also an exhausted solid yet rich in valuable components. Acid hydrolysis allows a practically total solubilization of this exhausted biomass which includes some amino acids, monosaccharides, and different degradation byproducts. This mixture of components could be useful as culture media for microorganisms able to accumulate polyhydroxyalkanoates for bioplastic production. However, as first step, it is necessary to study the economic and environmental feasibility of this biorefinery alternative by techno-economic and life cycle analysis (TEA and LCA), identifying the principal hotspots of this fractional recovery idea.

The production of peptides and PHAs from microalgae biomass grown on piggery wastewater was a feasible process with a net present value (NPV) of ~1,420,000 € after an investment cost of ~4,863,000 €, and a global warming impact (GW) of 294 kg CO_2 eq/m^3 of microalgae biomass (95% humidity), although the TEA identified various critical points. These including the high cost of centrifuges (65% of the total equipment cost), the high production cost of the biomass produced in open photobioreactors feed with wastewater, the low PHAs content in the initially selected microorganisms and the high electricity requirement for ultrasonication. Improving these identified hotspots, it was possible to enhance the economic sustainability of the proposed process up to 141% (by reducing of the number of centrifuges), 91% (by reducing the biomass cost up to 0.77 €/kg_{CDW}) and 84% (by increasing the PHAs content in the microorganisms up to 70%).

Likewise, the LCA found that electricity was the principal contributor to the global warming impact (50%) due to the high fossil origin of electricity production in Spain. Thus, this limitation should be studied and optimised to achieve a more sustainable biorefinery process in the future, with GW reduction up to 250 kg CO_2 eq/FU after the optimization and reduction of the electricity requirements of ultrasonication extraction which is the main contributor to the electricity necessities of the process. These results showed the importance of carrying out a techno-economic and life cycle analyses to identify the weaknesses of a biorefinery process in an early stage, helping to avoid high economic and environmental costs before implementation of an emerging technology like microalgae biorefinery.

Based on all these results, future research could be focus on the following points:

- Optimization of the solvent extraction step of biopesticide production, physicochemical analysis of the biopesticide product and laboratory and agronomic studies of biopesticide efficiency.
- Life cycle analysis of biopesticide production from biomass grown on piggery wastewater.
- Optimization of operational parameters (hydrolysis time, energy, type of enzyme, biomass/enzyme ratio, temperature…) of the UAEE.
- Study and develop an analytical method of chemical analysis of veterinary antibiotics in the hydrolyzates obtained.
- Investigation and optimization of the main hotspots of the proposed biorefinery process for peptide and PHAs production.
- Optimization of methods to separate monosaccharide and peptides from hydrolyzates and to purify the recovered peptides.
- Optimization of methods to recovery and purify PHAs from the microorganisms.

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Elena María Rojo de Benito (Valladolid, 1992) holds a degree in Chemical Engineering in 2015 from the University of Valladolid (Spain) and a MSc in Environmental Engineering in 2016 from the University of Valladolid (Spain). After completion of their university studies, he started working as a specialist engineer in "Confederación Hidrográfica del Duero", where she stayed for 3 years. In 2019, Elena joined the Institute of Sustainable Processes (ISP) of the University of Valladolid as a predoctoral investigator to develop her PhD thesis under the supervision of Dra. Silvia Bolado Rodríguez and a PhD contract by "Ministerio de Ciencia, Innovación y Universidades".

Her research focused on the valorization of microalgae biomass from piggery wastewater treatment to obtain proteins, carbohydrates and other bioproducts such as biostimulants for agriculture or bioplastics. This work was supported by various projects: PURASOL project titled "Caracterización y valorización fraccionada de biomasa algal crecida en plantas de tratamiento de purines", PROPHACTION project titled "Recuperación de proteínas y producción de PHA a partir de biomasa generada en plantas de tratamiento de aguas residuales", and GREENFARM project titled "Sustainable production of agricultural biostimulants and biopesticides using agricultural waste", all of them founded by "Ministerio de Ciencia, Innovación y Universidades".

During her PhD, she carried out two research stay for 2 months in University of Almería (2022, Spain) under the supervision of Dr. Francisco Gabriel Acién Fernández and for 3 months in Politecnico di Milano (2023, Italy) under the supervision of Dr. Giovanni Dotelli.

Publications

Rojo, E.M., Rossi, S., Bolado, S., Stampino, P.G., Ficara, E., Dotelli, G. (2024) Life cycle assessment of biostimulant production from algal biomass grown on piggery wastewater. Science of the Total Environment, 907, 168083.

Rojo, E.M., Molinos-Senante, M., Filipigh, A.A., Lafarga, T., Acién Fernández, F.G., Bolado, S. (2023). Agricultural products from algal biomass grown in piggery

wastewater: A techno-economic analysis. Science of the Total Environment, 887, 164159.

Rojo, E.M., Filipigh, A.A., Bolado, S. (2023). Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass. Process Safety and Environmental Protection, 174, 276-285.

Rojo, E.M., Piedra, I., González, A.M., Vega, M., Bolado, S. (2021). Effect of process parameters on the valorization of components from microalgal and microalgal-bacteria biomass by enzymatic hydrolysis. Bioresource Technology, 335, 125256.

Reviews

Moldes, D., Rojo, E.M., Bolado, S., García-Encina, P.A., Comesaña-Gándara, B. (2022). Biodegradable solvents: A promising tool to recover proteins from microalgae. Applied Sciences, 12, 2391.

Book chapters

Rojo, E.M., Filipigh, A., Moldes, D., Vega, M., Bolado, S. (2022). Chapter 4: Potential of microalgae for protein production in Algal biorefineries and the circular bioeconomy. Edited by Sanjeet Mehariya, Obulisamy Parthiba, Karthikeyan, Shashi Kant Bhatia, 91 – 132.

Rojo, E.M., Carmona, A., Soto, C., Díaz, I., Fernández-Polanco, M., Palacio, L., Muñoz, R., Bolado, S. (2021). Chapter 2: Environment and material science technology for anaerobic digestion-based circular bioeconomy in Circular bioeconomy – Current developments and future outlook. Edited by Ashok Pandey, Rajeshwar Dayal Tyagi and Sunita Varjani, 25 – 55.

Conferences contribution

Rojo, E.M., Hurtado, M., Filipigh, A.A., Ciardi, M., Acién-Fernández, F.G., Bolado, S. “Effect of veterinary antibiotics and heavy metals in the composition and valorization of a consortium of microalgae and bacteria” in 6th IWA International Conference on Eco-Technologies for Wastewater Treatment, 26 – 29 June 2023, Girona, Spain (short presentation).

Filipigh, A.A., Rojo, E.M., Bolado, S. “Protein and carbohydrate recovery from secondary sludge biomass generated in wastewater treatment plant” in 6th IWA

International Conference on Eco-Technologies for Wastewater Treatment, 26 – 29 June 2023, Girona, Spain (poster).

Rojo, E.M., Zambrano, J., García-Encina, P.A., Irusta, R., Jiménez, J.J., Hernández, F., Botero, A.M. "Mechanisms involved in veterinary antibiotics removal by microalgae-bacteria consortia" in 6th IWA International Conference on Eco-Technologies for Wastewater Treatment, 26 – 29 June 2023, Girona, Spain (poster).

Pila, A.N., Rojo, E.M., Filipigh, A.A., Dagnino, P., Bolado, S. "Use of hydrolyzates from waste microalgal biomass for bioplastics production by Paracoccus denitrificans: A preliminary study" in 11th World Congress of Chemical Engineering, 4 – 8 June 2023, Buenos Aires, Argentina (poster).

Filipigh, A.A., Rojo, E.M., Bolado, S. "Protein and carbohydrate recovery from secondary sludge biomass generated in wastewater treatment plant" in 2nd Greenering International Conference, 21 – 23 March 2023, Valladolid, Spain (short presentation).

Rojo, E.M., Filipigh, A., Molinos-Senante, M., Fernández-Sevilla, J.M., Acién-Fernández, F.G., Bolado, S. "Agricultural products from algal biomass grown in piggery wastewater: A techno economic analysis" in 2nd Greenering International Conference, 21 – 23 March 2023, Valladolid, Spain (poster).

Rojo, E.M., Hurtado, M., Ciardi, M., Bolado, S. "Effect of veterinary antibiotics and heavy metals in the composition and valorization of a consortium of microalgae and bacteria" in 10ª Jornada de Doctorandos, 1 December 2022, University of Valladolid, Spain (poster).

Rojo, E.M., Filipigh, A.A., Bolado, S. "Comparison of technologies for recovery of proteins and carbohydrates from microalgal biomass grown in piggery wastewater" in 13th IWA Specialist Conference on Wastewater Ponds and Algal Technologies, 3 – 6 July 2022, Melbourne, Australia (short presentation).

Rojo, E.M., Filipigh, A.A., Bolado, S. "Valorization of macrocompounds from a consortium of microalgae and bacteria by chemical hydrolysis and novel extraction methods" in 9ª Jornada de Doctorandos, 2 December 2021, University of Valladolid, Spain (poster).

Rojo, E.M., Vega, M., Bolado, S. "Recovery of proteins from biomass grown in wastewater treatment photobioreactors" in 3rd International Conference on Bioresource

Technology for Bioenergy, Bioproducts & Environmental Sustainability, 14 – 17 May 2021, Lake Garga, Italy (presentation).

Rojo, E.M. "Sequential valorization of microalgal biomass grown in pig manure treatment plants" in 8ª Jornada de Doctorandos, 2 December 2020, University of Valladolid, Spain (short presentation).

Rojo, E.M., Piedra, I., Vega, M., Bolado, S. "Effect of operational parameters and culture media on the solubilization of microalgal components by enzymatic hydrolysis" in Online Conference on Biogas Production and Digestate Valorization, 17 – 18 November 2020, Madrid, Spain (short presentation).

Rojo, E.M. "Characterization and fractional valorization of algal biomass grown in pig manure treatment plants" in 7ª Jornada de Doctorandos, 2 December 2019, University of Valladolid, Spain (short presentation).

Co-supervision

Degree Thesis: Rodrigo Beltrán Cortijo, student of BSc in Chemical Engineering, academic year 2022/2023. "Diseño de un proceso de extracción mediante ultrasonidos y filtración tangencial para la valorización de biomasa algal crecida en aguas residuales de purines". University of Valladolid, Spain.

Master Thesis: María Hurtado Gutiérrez, student of MSc in Advanced Techniques in Chemistry. Chemical Analysis and Quality Control, academic year 2021/2022. "Influencia de fármacos veterinarios y metales pesados en la valorización de biomasa algal crecida en aguas residuales de purines". University of Valladolid, Spain.

Degree Thesis: Jaime Moncada del Val, student of BSc in Chemical Engineering, academic year 2021/2022. "Diseño de una planta de recuperación de proteínas a partir de biomasa algal generada en una planta de purines". University of Valladolid, Spain.

Master Thesis: Ángel Alejandro Filipigh, student of MSc in Advanced Techniques in Chemistry. Chemical Analysis and Quality Control, academic year 2020/2021 "Valorización de un consorcio de microalgas y bacterias: comparación de métodos convencionales y nuevos métodos de extracción asistida". University of Valladolid, Spain.

Master Thesis: Irene Piedra Sola, student of MSc in Advanced Techniques in Chemistry. Chemical Analysis and Quality Control, academic year 2019/2020. “Efecto de los parámetros operacionales y las condiciones de cultivo en la valorización de microalgas mediante hidrólisis enzimática”. University of Valladolid, Spain.

Teaching

Academic year 2022/2023: Lecturer of “Cálculo y diseño de operaciones de separación” in Chemical Engineering Degree. University of Valladolid, Spain. 0.5 ECTS.

Academic year 2022/2023: Lecturer of “Tecnología Ambiental y de Procesos” in Engineering Degree. University of Valladolid, Spain. 2 ECTS.

Academic year 2021/2022: Lecturer of “Tecnología Ambiental y de Procesos” in Engineering Degree. University of Valladolid, Spain. 2 ECTS.

Academic year 2020/2021: Lecturer of “Tecnología Ambiental y de Procesos” in Engineering Degree. University of Valladolid, Spain. 2 ECTS.

Academic year 2019/2020: Lecturer of “Tecnología Ambiental y de Procesos” in Engineering Degree. University of Valladolid, Spain. 2 ECTS.

Specialized courses

Course “Programación Orientada a Objetos y Funcional con Python”. University of Valladolid, 29 Nov 2024. 2 hours.

Course “Taller de Introducción a Python”. University of Valladolid, 29 Nov 2024. 2 hours.

Course “Realización de figuras de calidad para artículos científicos”. University of Valladolid, 25 May 2023 – 29 June 2023. 8 hours.

Course “Excel para investigadores”. University of Valladolid, 24 November 2022. 2 hours.

Course “Elaboración de una propuesta de proyecto”. University of Valladolid, 18 November 2022. 2 hours.

Course “Introducción al diseño de experimentos”. University of Valladolid, 7 – 10 November 2022. 8 hours.

Course “GoToMarket: Developing a business case from your research project”. EIT Food and Council of Castilla y León, 4 October 2022. 10 hours.

Course “¿Cómo realizar una evaluación económica en proyectos de ingeniería?”. University of Valladolid, 24 June 2022. 4 hours.

Course “Principios básicos de cromatografía líquida de alta resolución (HPLC) y manejo del LC-2050C de Shimadzu”. Izasa Scientific, 20 – 21 January 2022. 14 hours.

Course “Análisis de ciclo de vida: Fundamentos y casos prácticos”. University of Valladolid, 21 October 2021. 5 hours.

Course “Curso de Inglés (Writing) para alumnos de la Escuela de Doctorado, nivel B2”. University of Valladolid, 3 March 2021 to 2 June 2021. 50 hours.

Course “Formación en Comunicación y Soft Skills”. University of Valladolid, 20 November 2020. 8 hours.

Course “Abstracts y artículos en inglés (ciencias, ciencias de la salud, ingeniería y arquitectura”. University of Valladolid, 8 – 20 June 2020. 16 hours.

Course “Curso de Inglés (Speaking) para alumnos de la Escuela de Doctorado, nivel B2”. University of Valladolid, 2 March 2020 to 8 June 2020. 50 hours.

Course “Writing skills”. Researcher academy, Elsevier. 27 April 2020.

Course “Funding”. Researcher academy, Elsevier. 20 April 2020.

Course “Fundamentals of manuscript preparation”. Researcher academy, Elsevier. 20 April 2020.

Course “Microalgae biorefineries as multi-product integrated biorefineries: a course on combined modelling and experimental approaches”. University of Valladolid, 21 – 22 January 2020. 6 hours.

Course “Taller práctico sobre Técnicas Analíticas Físico-Químicas e Instrumentales”. University of Valladolid, November 2019. 8 hours.

Course “Iniciación a la escritura y publicación de artículos científicos (ingenierías y arquitectura)”. University of Valladolid, 17 December 2019. 4 hours.

Course “Técnicas Básicas del Laboratorio en investigación biomédica 19-20”. University of Valladolid, 10 – 13 December 2019. 15 hours.