Journal of Cleaner Production 285 (2021) 125461

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Early-stage sustainability assessment of enzyme production in the framework of lignocellulosic biorefinery

Sara Bello [a, *], Noelia Pérez [a], Jan Kiebist [b], Katrin Scheibner [b], María Isabel Sánchez Ruiz [c], Ana Serrano [c], Ángel T. Martínez [c], Gumersindo Feijoo [a], Maria Teresa Moreira [a]

[a] Department of Chemical Engineering, CRETUS Institute. Universidade de Santiago de Compostela, 15782, Santiago de Compostela, Galicia, Spain
[b] JenaBios GmbH, Löbstedter Str. 80, 07749, Jena, Germany
[c] Centro de Investigaciones Biológicas Margarita Salas, CSIC, 28040, Madrid, Spain

ARTICLE INFO

Article history:
Received 11 May 2020
Received in revised form
28 October 2020
Accepted 7 December 2020
Available online 9 December 2020

Handling editor Cecilia Maria Villas Bôas de Almeida

Keywords:
Oxidative enzyme production
Life cycle assessment
Biorefinery
Early stage LCA
UPO
HMFO

ABSTRACT

The use and integration of enzymatic processes for the biotransformation of biomass within the biorefinery framework creates the need to confirm whether these novel production systems are in the route to environmental sustainability. In this study, the environmental profiles of the production of two oxidative enzymes, hydroxymethylfurfural oxidase (HMFO) from *Methylovorus* and unspecific peroxygenase (UPO) from *Chaetomium globosum* (*Cgl*UPO) for the enzymatic production of FDCA as precursor of bioplastics were analyzed. Laboratory-scale experiments allowed the identification of the consumption of energy, with over 80% share in every impact category for HMFO and chemicals and energy in *Cgl*UPO as primary hotspots of the systems. The results are transposed for HMFO when laboratory inventories were extrapolated to full scale processing, showing that impacts are attributed not only to energy demand but also to the use of chemicals required for the formulation of the culture medium. In terms of process units, the fermenter, where enzyme production takes place, corresponds to the stage that contributes the most to the environmental impacts, with a 57% share, followed by the downstream separation scheme (37%). Extrapolation of laboratory data to full-scale also represented a change in the relative difference of the impact per functional unit of 45% for *Cgl*UPO. The endpoint damage categories showed a significant reduction in their full-scale impacts to about half the burden. The analysis of the outcomes of the uncertainty analysis showed that the resource depletion category had the least dispersion of data, while the level of uncertainty is more relevant for human health, as it takes into account the combined effect of a larger number of impact categories and the processes involved. This study shows that, although being bio-based catalysts, the production of enzymes involves several steps which may incur in environmental impact. Thus, it is recommended that enzymes are carefully included within the system boundaries for their evaluation, since they could be the major hotspot in the biorefinery value chain. De-fossilization of the plastic industry will be possible with thoroughly optimized bio-transformations, with carbon-based media from residual resources, minimized use of chemicals and the implementation of energy integration measures.

1. Introduction

Every area of industrial production is in a trend to reach the sustainability goals set for Europe. The EU strategy for the bioeconomy envisages new value chains and the optimization of existing ones through decarbonization and the reduction of their environmental impacts. Biotechnology presents great opportunities for the development of production processes that generate high value-added products that meet basic environmental preservation objectives (Aguilar et al., 2019). The approach based on biocatalysis, enzymatic improvement, metabolic engineering and synthetic biology in biorefineries is of special interest, as the usage of renewable raw materials is combined with green and efficient means of production that are expected to yield lower environmental impacts than chemical ones (Choudhury, 2020).

Among potential renewable resources, lignocellulosics are

* Corresponding author.
E-mail address: sara.bello.ould-amer@usc.es (S. Bello).

https://doi.org/10.1016/j.jclepro.2020.125461
0959-6526/

receiving considerable attention due to their abundance and composition -cellulose, hemicellulose and lignin- from which biofuels and high value-added chemicals can be obtained (Martínez et al., 2009). In this field, several studies have been developed to gear research towards the production of specific enzymes for the biotransformation of these components.

Commonly used enzymes in lignocellulosic biorefineries include cellulases, hemicellulases, monooxygenases, ligninases, amylases, pectinases, lipases and proteases (Choudhury, 2020) that are typically applied in biomass pre-treatment, enzymatic hydrolysis, saccharification and fermentation processes (Álvarez et al., 2016; Maclean and Spatari, 2009). However, other research in lignocellulose exploitation is the conversion of diverse valuable compounds originated from chemical or enzymatic pre-treatment of biomass. This is the case of 5-hydroxymethylfurfural (HMF), a furan compound obtained from sugars (generally fructose) derived from cellulose hydrolysis (Van Putten et al., 2013). As it is formed by an aromatic ring and two functional groups (hydroxymethyl and aldehyde), HMF is an interesting option to be a starting material for chemical applications. In this way, special attention has been paid to its oxidation, as it provides convenient synthetic pathways for the production of chemical building blocks for the polymer industry. One of the main HMF-derived compounds is 2,5-furandicarboxylic acid (FDCA) which is listed as one of the 12 sugar-based platform chemicals of interest by the US Department of Energy since it can co-polymerize with diols producing polyethylene furanoate (PEF), a promising substitute of petroleum-derived polyethylene terephthalate (PET) (Huang et al., 2016).

Most of the methods described in the literature for the oxidation of HMF into FDCA are usually based in the use of metal catalysts such as carbon or alumina-supported platinum or platinum-supported lead (Tong et al., 2010). However, although some of them are promising, all of these methods are typically performed at elevated temperatures and pressures and with low selectivity, rendering the process expensive and polluting. Because of this, there is an increasing interest in shifting away from heterogeneous and chemically based catalysis processes, analyzing the production pathways of FDCA from the enzymatic perspective. To this end, several oxidative enzymes have been reported as promising alternatives with high potential to achieve such transformation (Sajid et al., 2018). Through these oxidative enzymes and their potential scalability for FDCA production, the biocatalytic routes differ from the more conventional methods reducing to a maximum the environmental and economic impact. Enzymatic catalysis shifts the oxidation process to milder conditions, higher selectivity to the production of furan-based compounds and the use of less harsh chemicals. This, in combination with the fact that the substrates required for enzyme production may be, in many cases, derived from biomass or biorefinery wastes, environmental impact results are expected to have an even further decrease (Domínguez de María and Guajardo, 2017; Yuan et al., 2020).

Hydroxymethylfurfural oxidase (HMFO) is able to produce FDCA from HMF (Dijkman and Fraaije, 2014). Unspecific peroxigenase (UPO) catalyzes the limiting-step for FDCA production for most oxidases (Serrano et al., 2019), the oxidation of formylfurancarboxylic acid (FFCA) into FDCA with hydrogen peroxide as co-substrate; its use in combination with oxidases such as aryl alcohol oxidase or galactose oxidase lead to FDCA production (Carro et al, 2015, 2018; Karich et al., 2018). This article aims to answer several research questions in the field of oxidative enzymes for the bioproduction of FDCA, using HMFO from *Methylovorus* expressed in *E. coli* (Viñambres et al., 2020) and *Cgl*UPO expressed in *Chaetomium globosum* (Kiebist et al., 2017) as model enzymes for this process.

Nonetheless, despite the boost in formulating a variety of enzymes targeted to participate in quite specific processes, there are challenges that must be addressed when it comes to the application of enzymes in full-scale processes. The overall efficiency of the process in technical and economic terms must be confirmed, considering the requirements of the enzymes and, of course, the environmental assessment of the enzymatic process.

Life cycle assessment (LCA) is a methodology aimed at analyzing the whole life cycle of a process or a product by means of gathering data regarding its input and output flows and transforming them into environmental impacts. These impacts, in the form of several descriptors -global warming, eutrophication, toxicity, ozone depletion- to name a few, allow to provide global view of the process under study. However, there is little to no knowledge on how the production of enzymes may affect the environmental performance of enzymatic transformations, as most of the studies about LCA found in the literature include the usage of enzymes as an anecdotal part of the whole assessment (Raman and Henning, 2013). In most cases, inventorying a complete list of flows involved in the production of enzymes, is a time-consuming process, in which most authors do not put the focus when environmentally analyzing enzymatic processes in biorefining routes. Olofsson et al. (2017) present the environmental study of ethanol production with a focus on the differences found when considering an on-site versus off-site production of cellulase enzymes. While they are able to provide detailed inventories for their simulated production of on-site enzymes, their ability to gather inventories for the externalized production was very limited due to the scarce availability of data in literature and the aggregated nature of the information. The study provides the environmental results by means of greenhouse gas emissions, concluding that off-site enzyme production achieved significantly higher impacts, considering that reported data regarding enzyme dosage as well as their production present great uncertainty. These gaps in the evaluation of enzyme production processes may lead to incur in errors in the environmental evaluation of enzymatic bio-transformations (e.g. biofuel production). Likewise, Hong et al. (2013) provide the environmental impact of biofuel production in which cellulases are accounted for by means of the global warming indicator. When calculating and projecting the impacts of enzymes the main highlighted challenges are the proprietary or non-disclosable character of the majority of available data with regards to enzymes, the wide variety of available enzymes, enzyme cocktails and production methods, and the experimental scale of many of the existing production processes. These challenges should be addressed in further research, to which this study aims to contribute (Nielsen et al., 2007).

As other scientific studies state, the transparency in inventories, which fail to mention enzyme inputs in some cases, do not explore into whether available datasets in databases are applicable to the specific characteristics of the study or even fail to include them in the inventory pleading that they are used in a very small quantity and cut-off rules apply, make the effect of enzymes use in biotransformations debatable (Maclean and Spatari, 2009). Enzymes have been known to have very high production costs (Klein-Marcuschamer et al., 2012), which may lead to believe that their environmental contributions are not negligible, and furthermore relevant to any system. Considering the possibility of recycling and reusing enzymes to reduce their high cost and environmental impact may be key in many systems (Cheng et al., 2019). However, whether reuse is applicable or not depends on the specific type of enzymes under assessment, and their utilization objective. In all, this implies that including them within the boundaries of LCA studies as part of the foreground processes, rather than background processes should shed light into the missing gaps and provide an in depth analysis considering all sensitive factors to their production

and utilization (i.e. externalization of their production, reuse and recycle, scalability, production yield).

Cellulase is one of the most studied enzymes in the biorefinery framework, and yet, the available LCAs in literature do not focus on detailing the value chain of its production with disaggregated inventories or evaluating a range of impact categories relevant to the study besides global warming potential (also depicted as greenhouse gas emissions, climate change, etc.). Moreover, very few studies assess endpoint impact categories or uncertainty of the dataset. Furthermore, as laboratory scale production processes are not usually under the scope of LCA application, this work will serve as basis for further research on the upscale and deployment of oxidative enzymes, as it aims to provide the environmental weaknesses and advantages of the production of said enzymes (i.e. HMFO and UPO) and their areas of improvement. The specific objectives of the study are:

1) To evaluate the robustness and reliability of laboratory-scale process evaluation using LCA and the effect of scale on the enzyme production process. To do so, the laboratory inventory data of enzyme production will be analyzed through a prospective perspective in LCA and provide an estimated full-scale outlook.
2) To analyze which are the environmental hotspots of enzyme production, considering both midpoint and endpoint indicators, in order to study the direct consequences to the environment and the damage produced to the three main areas of protection (human, ecosystems and resources). This will serve as basis to depict what are the optimization steps needed to improve the sustainability of such processes.
3) As the Ecoinvent database (Wernet et al., 2016), one of the most important datasets in the field of LCA practitioners, does not provide detailed inventories for the production of enzymes, this work is intended to represent an ex-ante LCA contributing to the early stages of enzyme database compilation and evaluation of environmental results.

2. Materials and methods

The evaluation of the environmental burdens for enzyme production was performed by implementing the methodology of attributional LCA, described through the ISO 14040 and ISO 14044 standards (ISO 14040, 2006; ISO 14044, 2006). In this study, the compulsory stages for the evaluation of processes or products through LCA were implemented: goal and scope definition, life cycle inventory, life cycle impact assessment and results interpretation stages.

2.1. Goal and scope definition

The production of two enzymes, HMFO and *Cgl*UPO was assessed by means of LCA with the objective of determining the main hotspots of their production process. The enzymes under study are used in oxidative reactions for the conversion of HMF to FDCA and of FFCA to FDCA. The functional unit of the study was defined as the enzyme activity (measured with vanillyl alcohol for HMFO and veratryl alcohol for *Cgl*UPO) achieved at the gate of the process, expressed in units (1 unit). The results of this analysis introduce a starting point for the evaluation and optimization of enzyme production processes, evaluated from an early stage perspective with primary data at laboratory scale. The environmental assessment was performed under a holistic perspective, including the inventory, midpoint results and an analysis of the damage categories. A streamlined upscale, in which energy was considered the key factor, was included to analyze the preliminary robustness of the environmental assessment of laboratory processes through LCA.

2.2. Production system and system boundaries

The production process for HMFO and *Cgl*UPO follows a standard biotechnological process sequence, which includes the pre-inoculum, inoculum, fermentation and downstream stages. The generic boundary of the system is presented in Fig. 1.

2.2.1. HMFO production

The pre-inoculum phase consists of the growth of the cell culture in a Petri dish with Luria-Bertani (LB) medium with bacteriological agar and antibiotics. This was transferred to a flask in which the inoculum is grown, again with LB medium and antibiotics. The inoculum was then transferred to the bioreactor with a volume of 25 L, where the production of the enzyme occurs in two phases: cell growth and induction of protein expression. The bioreactor and its contents were sterilized with an autoclave. The fermentation was aerated and agitated during the whole reaction time, at 37 °C during the growth phase and at 16 °C for the induction phase. The overall batch time of the production process is 120 h. The downstream scheme includes two microfiltration units with an intermediate freezing stage, as well as an ultrafiltration with an output of 7000 units.

2.2.2. CglUPO production

The inoculum for *Cgl*UPO production was fed with a medium containing sodium chloride, malt extract and agar-agar. The seed fermentation was transferred to a fermenter with an operating volume of 6 L for 672 h with a culture medium composed of glucose, peptone and yeast extract. The fermenter and its contents were sterilized with an autoclave. The fermenter was agitated, aerated and kept at 24 °C. The downstream processing includes a vacuum pump, centrifugation, microfiltration, ultrafiltration, freezing, cooling and a chromatography unit. Each batch yields 1200 units.

2.3. Life cycle inventory (LCI)

Input and output inventories were provided as primary data flows from the laboratory experiments. The energy consumption of the equipment was calculated based on the operating time and the maximum power consumption of each piece of equipment. The results from the LCI phase are presented in Table 1 for HMFO and Table 2 for *Cgl*UPO. The inventory data is specified per batch of production, which will be normalized to the functional unit in the life cycle impact assessment stage of the study.

As an approach to validate and analyze the LCA results obtained from laboratory experiments, a simplified scale-up approach has been adopted in which the electricity consumption was the main concern. The production was scaled to 100 m^3 by extrapolation of the inputs and outputs. The target volume was considered a feasible production volume in biotechnology operations and fermentations involving enzyme production. Regarding electricity consumption, several simulation case studies for bioprocesses have been analyzed, retrieving typical energy consumption values for common unit operations: agitation in a fermenter, steam demand for heating, cooling water demand, aeration, microfiltration, ultrafiltration, centrifugation and vacuum filtration.

The updated data and process descriptors for the two analyzed processes are presented in Table 3 for HMFO and Table 4 for *Cgl*UPO. The mass conversion factor allows extrapolating the laboratory scale inventories to the hypothetical production volume of 100 m^3.

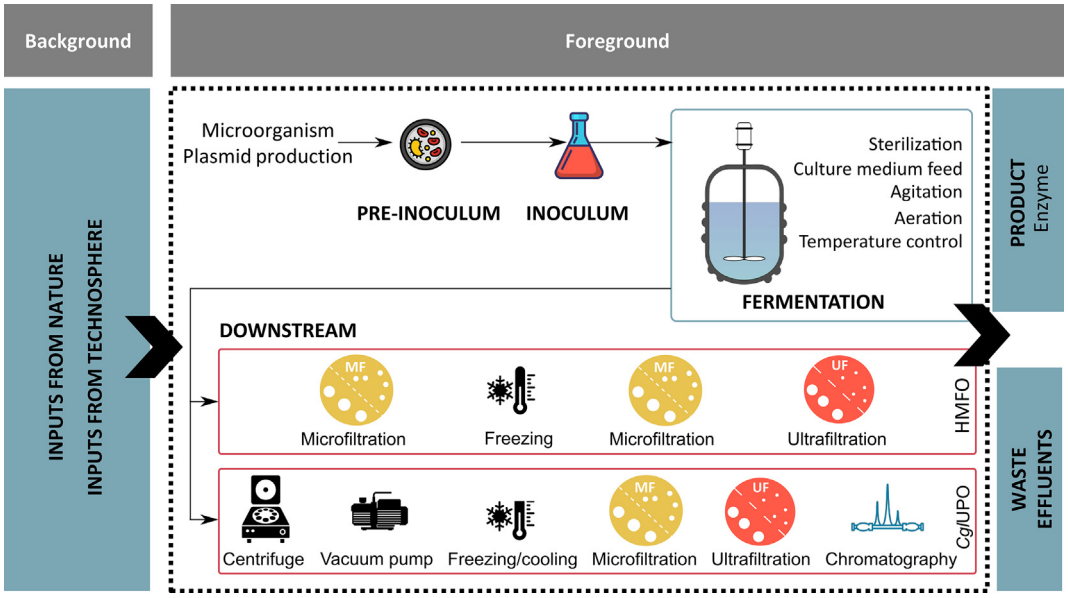

Fig. 1. Generic cradle-to-gate system boundaries for the production of HMFO and *Cgl*UPO enzymes at laboratory scale.

Although it cannot be predicted, it was considered that the enzyme activity produced in a large-scale process would increase in direct proportion to the production volume.

2.4. Assumptions and limitations

The main assumptions of the study were related to limitations in data availability. The plasmid, cells and biotin used in the pre-inoculum, were considered to have a negligible impact on the system under study. For the chemicals that were not available in the Ecoinvent 3.5 database, either other chemicals with equivalent characteristics were considered or bibliographic inventories were implemented in the software. Tryptone and peptone were replaced by soybean meal, available in Ecoinvent 3.5 (Delgove et al., 2019). Yeast extract and malt extract were substituted by protein feed and polyether sulfone was included in the LCA as polycarbonate. The inventory for bacteriological agar was retrieved from the production of carrageenan (Ghosh et al., 2015). The inventory for the production of antibiotics (ampicillin, chloramphenicol, zeocin) was considered as that for the production of Penicillin V (Harding, 2008). For IPTG (Carlsson et al., 1991) and tris(hydroxymethyl) aminomethane (Bourguignon et al., 1980), it was considered that the best approach was to build inventories based on stoichiometric ratios, which means that energy consumption was not included.

The electric mix of a country is the electricity retrieved from the grid as available in each country. This implies that depending on the distribution of production by source (e.g. coal, wind, solar, nuclear, etc.) and the imports/exports in each country, the carbon footprint of each electricity mix will change. For this study, the country in which each enzyme was produced was the one selected and from which the electricity was retrieved for each of the production processes. The German electric mix from the Ecoinvent database was selected for the energy consumption of *Cgl*UPO production process, while for HMFO production the electricity was sourced from the Spanish electric mix. No transport processes were considered for the foreground or background systems.

Off-gas emissions (i.e. CO_2) were excluded from the inventories of the system since they are of biogenic origin. This entails that they are derived from the biomass-based carbon introduced in the system (e. g. in the form of nutrients for the fermentation medium). In LCA calculations, biomass-derived carbon emissions are assumed to be neutral since it is considered that the carbon intake, at the end of its life cycle will be released back to the atmosphere and absorbed for plant growth. Neutrality in the emissions is depicted as a zero-characterization factor in the carbon footprint computation (Penman et al., 2006).

2.5. Methods

The environmental evaluation was based on the attributional approach, analyzing the processes under study with midpoint and endpoint impact categories. Midpoint categories are the environmental mechanisms linking the causes to the final effects (endpoint categories) in the cause-effect chain of environmental consequences (Goedkoop et al., 2009). The ReCiPe 1.1 (Huijbregts et al., 2016) hierarchist method was applied and implemented through the SimaPro 9.0 software. The Ecoinvent 3.5 database was used for the implementation and transformation of inventories for background processes in the system. The mid-level impact categories analyzed were global warming expressed in kg CO_2 eq (GW), ozone depletion in kg CFC11 eq (OD), ozone formation in kg NOx eq (OF), terrestrial acidification in kg SO_2 eq (TA), freshwater eutrophication in kg P eq (FE), marine eutrophication in kg N eq (ME), freshwater ecotoxicity in kg 1,4-DCB eq (FET), marine ecotoxicity in kg 1,4-DCB eq (MET), human toxicity in kg 1,4-DCB eq (HT), land use in m^2a crop eq (LU) and fossil scarcity in kg oil eq (FS). These are categories that can describe, overall, the environmental profile of biorefinery and enzymatic systems. Burden shifting of impacts when implementing bio-based scenarios may happen when de-fossilization is the main objective. Thus a representative range of indicators, describing relevant factors such as land use, eutrophication, acidification of soils, ozone-related categories and toxicity (due to the use of chemicals) should be addressed (Katakojwala and Mohan, 2021; Parajuli et al., 2015). The three endpoint categories were studied to have a generic descriptor of the main damage areas: human health (DALY), ecosystems quality (species·year) and resource depletion (USD, 2013). All midpoint impact categories were contributors to the endpoint results, including, together with the midpoint categories specified above and ionizing radiation in kBq Co-60 eq. (IR), particulate matter formation in kg PM2.5 eq.

Table 1
Inventory for the production of HMFO enzyme at laboratory scale per batch (25 L).

Item	*Amount*	*Units*
Stage 1: Pre-inoculum and inoculum		
Mass inputs		
Cells BL21 (DE3)pLys	1	g
Plasmid pET23b	1	g
Tryptone (LB media)	8.54	g
Yeast extract (LB media)	4.27	g
NaCl (LB media)	4.27	g
Bacteriological agar	0.81	g
Antibiotics	0.114	g
Distilled water	1854	g
Filters (PET)	6.86	g
Filters (polyether sulfone)	2.94	g
Autoclave water consumption	1000	g
Energy inputs		
Autoclave electricity consumption	3.46	kWh
Incubator electricity consumption	14.67	kWh
Residues		
Polyether sulfone (RSU)	9.8	g
Stage 2: Bioreactor		
Mass inputs		
Tryptone (LB media)	250	g
Yeast extract (LB media)	125	g
NaCl (LB media)	125	g
Antibiotics	3.35	g
Distilled water	27,000	g
Tap water	20,000	g
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	0.596	g
Energy inputs		
Bioreactor sterilization electricity	3.52	kWh
Bioreactor agitation electricity (induction)	13.14	kWh
Temperature maintenance bioreactor electricity	5.92	kWh
Bioreactor agitation electricity (growth)	0.72	kWh
Air compressor electricity	2.26	kWh
Air dehumidifier electricity	14.25	kWh
Recirculator electricity consumption	144	kWh
Stage 3: Downstream		
Mass inputs		
Tris (hydroxymethyl)aminomethane	12.1	g
HCl	5	g
NaOH	48	g
Distilled water	120	kg
Bleach	700	g
Energy inputs		
Peristaltic pump electricity	1.15	kWh
Agitator electricity	73.34	kWh
Freezer electricity consumption	6	kWh
Output		
HMFO	1.16 7000	g/batch units/batch
Residues		
Wastewater	29	L

(PMF), terrestrial ecotoxicity in kg 1,4-DCB (TET) mineral resource scarcity in kg Cu eq. (MS) and water consumption in m^3 (WC). An uncertainty analysis of the endpoint results was conducted through the Monte Carlo simulation module in the SimaPro software. The input parameters were considered as the available data uncertainties for the implemented activities (Ecoinvent 3.5 flows), which considered a default lognormal distribution. The Monte Carlo analysis was performed by setting the number of iterations to 2000 at a 95% significance level.

3. Results and discussion

3.1. Environmental study of enzyme production through the midpoint perspective

The produced enzymes, at laboratory scale achieved 1.16 g/batch (7000 units per 25 L batch) for HMFO and 1.82 g/batch (1200 units per 6 L batch) for *Cgl*UPO. Some authors have presented the production of HMFO at experimental scale (1 L batch) in which an activity of 120 units was achieved (Viñambres et al., 2020). If these results were to be extrapolated to a 25 L production volume, 3000 units would be obtained. Thus, the fermenter in this study, for the production of HMFO at 25 L volumes has doubled the production. In this study, it was assumed that through scale-up, the energy input to the system would be reduced, however, these results allow to argue that the productivity could be potentially increased through the use of a fermenter at larger scale -as indicated here-reducing the environmental impact per functional unit. In this way, the results presented here can be viewed as a conservative approach in terms of productivity.

The relative contributions depicting characterization results of the main activities in the production process of the two enzymes are presented in Fig. 2, both for laboratory scale and up-scaled inventories. For the laboratory experiments, during the production of HMFO, the largest contributor to the environmental impacts for all categories is electricity consumption. Electricity impacts are above 76% in all impact categories, while the use of chemicals for the culture medium or the consumption of soybean meal have minor contributions in LU and ME impact categories. This is a trend that is interconnected with the lack of energy optimization in laboratory experiments. Laboratory-scale processes are characterized by the focus on the design of a process or a product in an experimental environment, rather than on optimizing the use of resources.

To the contrary, the environmental profile of the *Cgl*UPO produced in the laboratory does not show one single hotspot. In this case, electricity consumption, chemical requirements (principally sodium chloride, tris(hydroxymethyl)aminomethane and ammonium sulfate) are presented as the most relevant activities. This trend is similar in all impact categories, except for ME, where the hotspot is glucose (72% contribution). In LU the impacts are, again, originated in processes related with cropping activities for the production of sugar feedstock and nutrients (glucose and soybean meal) which require vast extensions of land. Glucose from the Ecoinvent database is produced from maize grain, where cropping activities include the use of several nitrogen-based fertilizers, with nitrogen being the main contributor to the ME impacts. Glucose has minor impacts, in the range of 4–30% in the other categories. Ozone formation displays higher impacts for freezing (13%) and refrigeration (14%), which is not observed for other categories.

When processes were upscaled, as expected in the case of HMFO the fluctuation of the relative contributions is in the direction of reducing the share of electricity, which influences the performance of the enzymes and results in increased contributions from other activities such as chemical consumptions, energy for refrigeration or nutrients for the formulation of the fermentation broth such as yeast paste. The scale-up in the HMFO production resulted in electricity remaining the largest contributor to the impact in eight out of ten categories, its relative contribution being, however, lower than in the laboratory-scale scenario, with values ranging from 6 to 60% for ME and FE, respectively. Tryptone, simulated as soybean meal, contributes with 54% of the burdens to ME. The refrigeration activity contributes with 46% to the impacts in the OF category. While for HMFO the relative contribution of each activity and its distribution underwent a significant difference when comparing the laboratory scale and the scaled-up inventories, shifting the area

Table 2
Inventory for the production of *Cgl*UPO enzyme at lab-scale per batch (6 L).

Stage 1: Inoculum		
Item	Amount	Units
Mass inputs		
Inoculum (out of boundaries)	0.28	L
Malt extract	6.60	g
NaCl	0.30	g
Agar	6.60	g
Stage 2: Fermentation		
Item	***Amount***	***Units***
Mass inputs		
Glucose	235.20	g
Peptone	100.80	g
Yeast extract	25.20	g
Water for sterilization	5.00	L
Water	5.60	L
Energy inputs		
Sterilization	1.50	kWh
Agitation	1.66	kWh
Stage 3: Downstream		
Item	***Amount***	***Units***
Mass inputs		
Ammonium sulfate	663.00	g
Phenyl sepharose	25.00	g
Bis-Tris	37.00	g
Energy inputs		
Vacuum pump for filtration	0.028	kWh
Centrifugation	0.168	kWh
Ultrafiltration	0.13	kWh
Microfiltration	0.01	kWh
Freezing	18.00	kg day
Cooling	12.00	kg day
Chromatography	12.00	kWh
Output		
Peroxygenase (*Cgl*UPO)	1.82	g/batch
	1200	units/batch

Table 3
Inventory data for the scale-up production of HMFO.

Upscaled HMFO		
Volume	100	m^3
Batch Time	120	h
Fermentation time	76	h
Units	$28 \cdot 10^6$	U
Mass conversion factor	4000	
Energy consumption estimation		
Steam for sterilization	982.52	kg/batch
Agitation	16,875.32	kWh/batch
Cooling water	3540.39	kg/h
Aeration	290.97	kW/batch
Microfiltration 1	9.87	kWh/batch
Microfiltration 2	0.06	kWh/batch
Ultrafiltration	294.94	kW
Cooling chamber	125.00	kg day
Freezing chamber	20.00	kg day
Total energy consumption	17,471.16	kWh
Quantity of enzyme	4.67	kg
Units of enzyme/batch	28,000,000	U

of environmental interest. In this case, the conclusions that can be drawn from the environmental relative contributions at the laboratory scale are the need to optimize the use of electricity at every stage of production, from stirring and temperature maintenance to downstream unit operations to make the process feasibly scalable.

In the case of *Cgl*UPO, the upscaling of the electricity

Table 4
Scale up of inventory data for the production of *Cgl*UPO.

Upscaled *Cgl*UPO		
Volume	100	m^3
Batch Time	672	h
Fermentation time	672	h
Units	$20 \cdot 10^6$	U
Mass conversion factor	16,666.7	
Energy consumption estimation		
Steam for sterilization	2678.90	kg/batch
Agitation	149,213.37	kWh/batch
Cooling water	3540.39	kg/h
Aeration	308.11	kW/batch
Vacuum filtration	531.02	kWh/batch
Centrifugation	173.40	kWh/batch
Ultrafiltration	3560.07	kWh/batch
Microfiltration	9.72	kWh/batch
Freezing chamber	18	kg day
Cooling chamber	12	kg day
Total energy consumption	153,487.58	kWh
Quantity of enzyme/batch	30.30	kg
Units of enzyme/batch	20,000,000	U

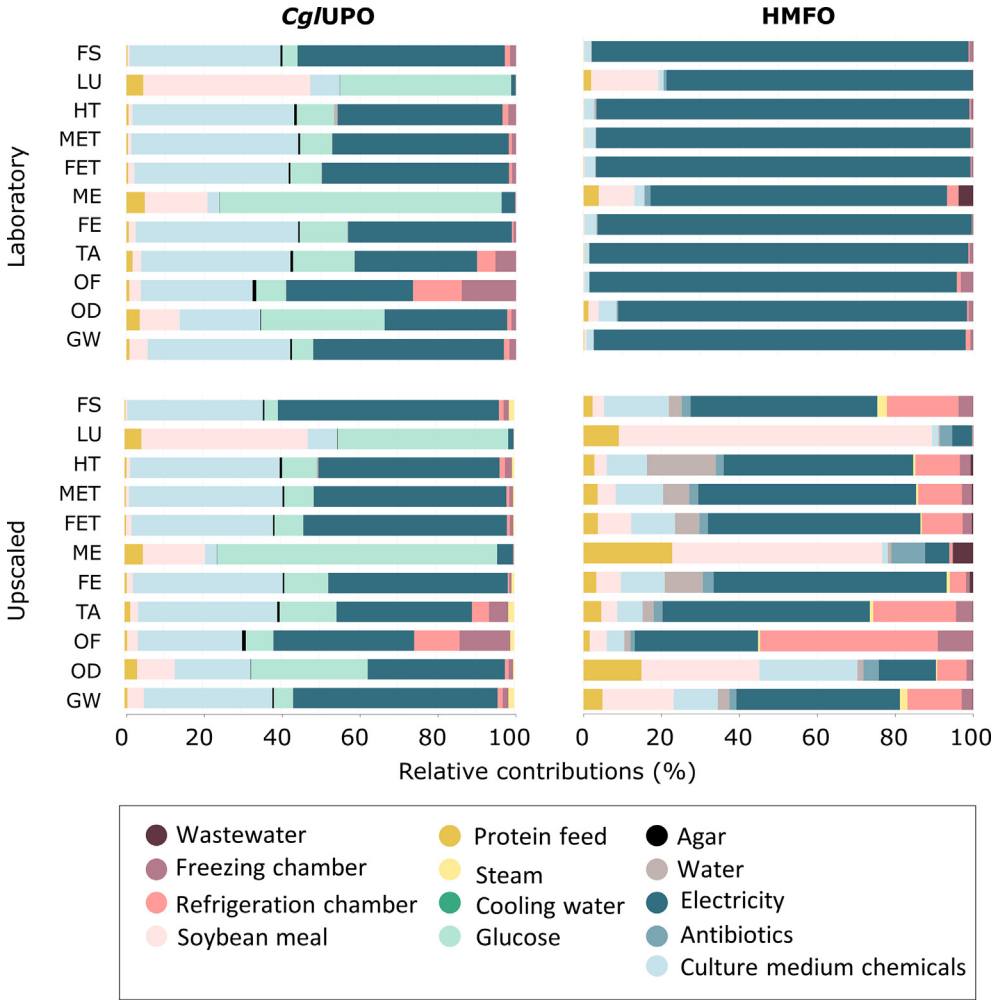

Fig. 2. Environmental profiles displaying relative contributions (%) of characterization results for *Cgl*UPO and HMFO enzymes for laboratory experiments and the estimated upscaled production per functional unit (1 Unit of enzyme). FS: fossil scarcity, LU: land use, HT: human toxicity, MET: marine ecotoxicity, FET: freshwater ecotoxicity, ME: marine eutrophication, FE: freshwater eutrophication, TA: terrestrial acidification, OF: ozone formation, OD: ozone depletion, GW: global warming.

consumption implied a proportionally lower value with a linear reduction. The relative contributions of the process remain almost unchanged to those of laboratory-scale production. While most relative impacts remain unchanged, the contribution from steam is incorporated. Steam is typically used in fermentation processes at larger scale, for activities such as sterilization.

The TA indicator is impacted by acidifying substances contributing to the change in pH of the soil. Main acidifying pollutants are ammonia, nitrates, nitrogen and sulfur oxides and sulfuric acid. In the production network of the *Cgl*UPO enzymes, TA has its root cause in glucose, ammonium sulfate and electricity. In the case of glucose, the production of starch from maize grain carries impacts related to the use of nitrogen fertilizers (ammonium nitrate) and the diffuse emissions from applying such fertilizers. For HMFO, the tree of contributions is mostly marked by electricity production by coal, to which the fuel in transoceanic ship transport was the most relevant background process in the chain.

In toxicity categories (FET and MET), HMFO displays, apart from electricity, which is the main contributor, protein feed, soybean meal and antibiotics as relevant contributors, which have background processes involving chemicals that raise the toxicity potential of the system. The same trend is seen in *Cgl*UPO, in which the chemicals from the background of nutrient production for the medium are the root cause for toxicity. In these enzymes, protein feed, (mostly affected by sulfuric acid in its background), tris(hydroxymethyl)aminomethane (with the effect of methanol in its value chain) and ammonium sulfate are the most polluting chemicals.

The use of electricity has the most prominent effect in the GW results in this study, being the main hotspot for all the evaluated enzymes. The attributed shares to the impacts are 49% and 95% for laboratory production of *Cgl*UPO and HMFO and 52% and 42% for the upscaled scenarios. In this study, German and Spanish electricity mixes were selected, yielding a high impact in the carbon footprint category, derived from the coal dependability in the overall generation of electricity, which is the form of electricity production with most carbon intensity (Tranberg et al., 2019). The use of electricity mixes with a higher share of renewables (i.e. Norway) would provide potential to diminish the impacts in this category, especially in cases in which the use of electricity is under optimized (i.e. laboratory production of HMFO). However, the geographic location of the production site for enzymes will most probably be located within the facility employing the produced enzymes. Energy optimization and integration should be the first step sought in order to reduce the carbon dependability of the system, which should lean into the use of energy produced within system boundaries, for instance through cogeneration systems exploiting biomass-based residues as a carbon abatement option (which would emit non-fossil carbon emissions). Heat production in the chemical industry was another major hotspot -specifically in the case of *Cgl*UPO- contributing to GW. Although chemicals are used in lower quantities, their impact is characterized by the energetic demand (heating systems) of their production. For such results, again, depending in lower nutrients for the culture medium for example agro-industrial residues (Pandey et al., 2000), can potentially curb the impact of chemicals from the petrochemical industry. Although having small deviations, the FS impact category parallels almost perfectly the behavior of the GW category. The root cause is the dependency on fossil fuels of the energy and chemicals selected in the system, which directly affects the carbon emissions of the system.

It is well known that the production of FDCA with oxidative metal catalysts is a feasible practice (Albonetti et al., 2015; Triebl et al., 2013). When analyzing the environmental results of these processes, it becomes evident that GW is a key impact category in their evaluation. Not only because of the need to decarbonize the platform chemical production sector, in a more global view, but also because it has been demonstrated that for oxidative catalytic processes in the production of FDCA, the energy consumption of the process and the use of lignocellulosic feedstocks for HMF production are the main hotspots. In both cases, they affect the GW category in a relevant manner (Isola et al., 2017).

On the other hand, although the presentation of the contribution results of each of the activities that are included within the process is interesting in the sense of analyzing the areas of improvement of each production route, the enzymes are produced with the sole purpose of contributing to the overall reduction of the impacts of the bioprocessing routes. For this, in this study, the GW results were analyzed for different potential enzyme loads in a hypothetical bioprocessing route. The enzyme loads were analyzed in a range of 1–333,000 activity units (Fig. 3).

Fig. 3 allows discerning whether the impact of the production of enzymes is low, high or very high in relation to the impact of the process using such enzymes. For instance, for HMFO results at laboratory scale, the environmental feasibility of the use of the enzyme as a catalyst for biotransformation, in terms of kg of CO_2 eq, will be determined by the overall impact of the production process (e.g. oxidation of HMF to FDCA with the use of HMFO or oxidation of FFCA to FDCA by *Cgl*UPO) and the enzyme load needed for such transformation. For the impact values in the enzymatic conversion process equal or higher than the GW impact for each enzyme load, the impact contribution of HMFO production would be very significant (red background in the graph) and the enzymatic transformation would be considered highly disadvantageous. When the enzyme contribution to the process is in the range of 40–100% (orange shades in the graph), the production of enzyme would be a relevant hotspot in the transformation, having to perform a substantial optimization of the consumables affecting the environmental results. For relative impacts below 40% (yellow and green areas in the graph), the use of enzymes may be considered feasible, always at the expense of performing a direct comparison with the conventional or non-enzymatic production process.

Fig. 3 also illustrates the main differences in the net value of GW impacts for laboratory inventories compared to their upscaled counterpart per functional unit. According to the results, the laboratory scale processes always present a higher GW than their upscaled counterparts. In the case of HMFO, the relative difference per functional unit displays a 97% decrease in the GW category when the process is upscaled for the studied unit loading range. However, the relative difference per functional unit is 45% for *Cgl*UPO. In this sense, it can be concluded that it is not reliable to evaluate the potential environmental impacts of enzyme production processes that are intended to be upscaled with data of experiments at smaller volumes. This is especially true, in this study, for HMFO enzyme, while *Cgl*UPO may experience lower errors if this procedure is followed. It was found that, since electricity was the main contributor to impact in laboratory scale processes, its potential optimization towards scalability of the processes, will indeed reduce substantially the impacts associated with the production of enzymes. The range of reduction is wide, as is the range of impacts that may be achieved in the GW category. Disclosing energy use as one of the most important aspects in upscale, for the reduction of impacts, is common in a wide range of bio-based systems (Carvalho et al., 2019), and in which, in many cases the bottom-up approach for the estimation of commonly missing data in LCI (e.g. energy consumptions) is a realistic approach (Parvatker and Eckelman, 2019).

Other studies have revealed the limitations of laboratory scale LCA results and the relevant differences when benchmarked with scaled-up results. This is a trend in the sectors of emerging

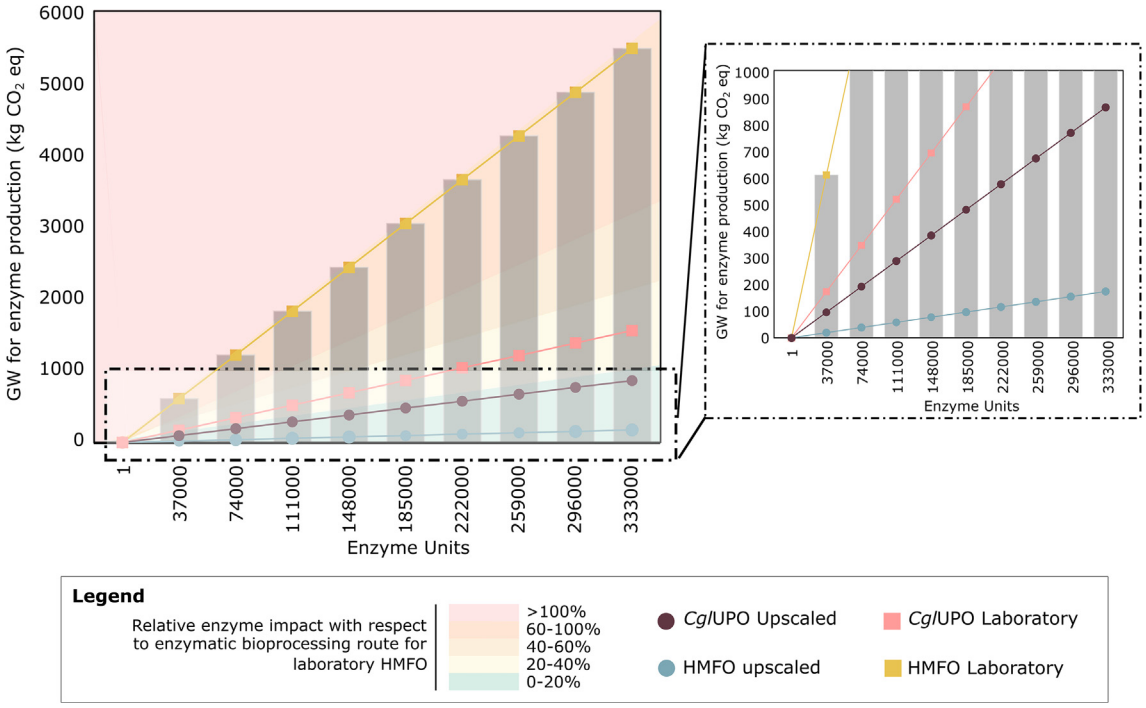

Fig. 3. Variability of the GW impact category characterization results (kg CO_2 eq.) per functional unit as a function of enzyme loading units. Results appear displayed for the two enzymes analyzed (HMFO, *Cgl*UPO) for both laboratory and upscaled inventories. The bars in grey display the maximum GW impact value among the included cases corresponding to laboratory HMFO.

technologies and products, which are still developed at low Technology Readiness Levels, for which primary data is not readily available and the processes are not considered mature. For instance, Gavankar et al. (2014) have analyzed the effect of the scale-up in the production of nanotubes, obtaining reduction values of 84–94% in a cradle-to-gate LCA and have detected, similarly to this study, the intensity of energy demand in smaller production volumes. Piccinno et al. (2018) analyzed the environmental results by means of LCA of the nanocellulose production process through the estimation of inventories at industrial scale, reporting the reductions per functional unit of the upscaled results when compared to laboratory production.

Fig. 4 shows the comparative evaluation for the two enzymes analyzed in this study in the two scenarios (laboratory and upscaled production). In the case of GW, the results were presented in Fig. 3. Regarding the rest of the impact categories evaluated, the comparative distribution maintains a similar trend to GW. The upscaled HMFO is the scenario with the lowest impacts in most of the considered categories, followed by the upscaled production of *Cgl*UPO, laboratory *Cgl*UPO and laboratory HMFO. This trend is not followed in ME, for which *Cgl*UPO at laboratory scale has the greatest impacts. The laboratory scale of HMFO presents a reduction of 23% of the impacts with respect to *Cgl*UPO production at the same scale. In the case of the upscale production, the enzymes differ in 74% of impacts, being HMFO the worse scenario.

The overall results of these enzymes, that can be potentially utilized for the same purpose (i.e. oxidation of HMF or FFCA to FDCA), and that present a wide range of differing impacts, show that including the production process of enzymes within the boundaries and scope of bioprocess environmental studies involving different enzymes is recommended when possible.

3.2. Environmental impact assessment though the endpoint perspective

Fig. 5 presents a perspective in which the damage pathways are analyzed through endpoint categories implementing the method previously described. The graph displays the endpoint results for three impact categories: human health, ecosystems quality and resource depletion for all the enzyme scenarios presented (laboratory and up-scaled inventories). The bar-graph additionally includes the relevance of each midpoint indicator within each endpoint category. The plots to the right side of the figure include the results of the Monte Carlo analysis uncertainty displayed through boxplots which show the median, first and third quartiles and the minimum and maximum values. The reason for presenting the Monte Carlo results for endpoint indicators is to take into account the aggregation of uncertainties when introducing additional considerations and assumptions into the calculations with the implementation of endpoint characterization factors. It is also relevant to understand the effect of uncertainty related to the inventories and how this uncertainty is changed by the effect of scale. The midpoint categories present cause-related indicators, while the endpoint results are more oriented towards the effect of the activities on the three main areas of environmental protection.

The human health impact category presented in Disability-Adjusted Life Years (DALY) represents the years of life lost or the years of disability due to diseases or accidents caused by the environmental consequences derived from the system under study (Huijbregts et al., 2016). In this study, as expected, the production of enzymes at lab scale presents, per functional unit, higher contributions to the human health indicator, while these values are reduced for the upscaled scenario. Continuing with the trend observed in the midpoint analysis, *Cgl*UPO experiences the least reductions in impact when upscaled. This is due to the lower

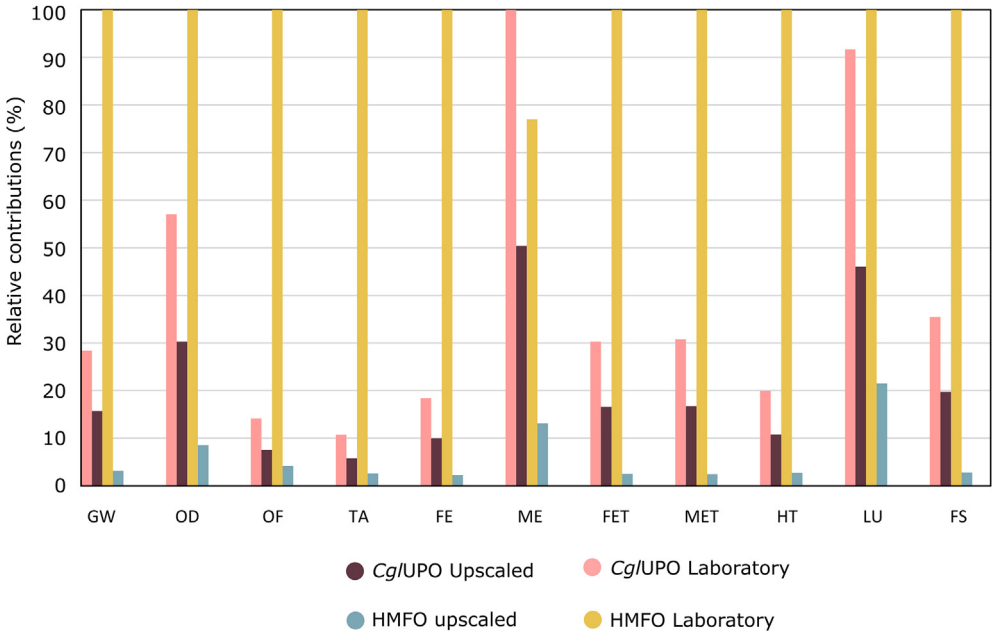

Fig. 4. Comparative evaluation of CglUPO and HMFO at laboratory and large-scale per functional unit (1 unit). FS: fossil scarcity, LU: land use, HT: human toxicity, MET: marine ecotoxicity, FET: freshwater ecotoxicity, ME: marine eutrophication, FE: freshwater eutrophication, TA: terrestrial acidification, OF: ozone formation, OD: ozone depletion, GW: global warming.

contributions from the energy consumption activity in the process compared to HMFO enzyme.

The human health impact category for the enzyme production system manifests relevant contributions mainly from the midpoint categories of GW, particulate matter formation (PMF) and HT. In the case of HT, the enzyme HMFO is the scenario with the least impacts for both scales analyzed. For *Cgl*UPO, the percentage contributions are 50%, 36% and 13% for GW, PMF and HT. For HMFO the contributions from the three main midpoint indicators have values of 35%, 55% and 8% for GW, PMF and HT. The greatest difference in the upscaled scenario occurs for HMFO, where the main contribution to human health is derived from GW with an 81% share. While the relative contributions of the midpoint categories do not experience a significant shift, it is notable that, in a direct comparison per functional unit, a potential energy optimization of the biotransformation (e.g., through upscale of the production volume) would decrease the effect in the category of human health. The impacts are reduced by a factor of 2 for *Cgl*UPO and 7 for HMFO.

Regarding the uncertainty of the values, the Monte Carlo simulation presents a way to perform a data validation analysis, in which the deviations in each scenario can be compared with their upscaled counterpart. The HMFO production at laboratory scale shows a major dispersion of the calculated endpoint impact, but also the greatest reductions in the uncertainty for human health. The dispersion of the results, characterized through the standard deviation, is reduced by 98.6% for HMFO, which depicts a great unreliability of the laboratory endpoint results for human health estimated for the enzyme, which can be addressed with upscaling procedures. The most significant results per functional unit for the human health impact category are those of the production of upscaled HMFO, presenting a standard deviation of $1.27 \cdot 10^{-9} \pm 1.37 \cdot 10^{-9}$ DALY which benchmarks the result with the least dataset dispersion. However, the results present a fairly high dispersion of the Ecoinvent dataset. In the case of *Cgl*UPO, the upscaled results present higher scattering than their laboratory counterparts in relative terms. The reductions of data dispersion in the upscaled scenario are not as relevant where one of the hotspots is the consumption of chemicals rather than electricity consumption. This is probably due to the higher effect of the use of chemicals in the human health impact category, which is a trend that can be observed for this enzyme, being also the process with the largest effect in the impact category under study. Also, higher reductions in the consumption of electricity, present higher reductions in the human health impact category. While HMFO presents contributions to human health that reach more than 95% in electricity use, CgI UPO presents a more distributed profile, with half of the impacts on human health coming from electricity consumption, about 25% from ammonium sulfate, 12% from tris(hydroxymethyl)-aminomethane, 5% from soybean meal and 5% from glucose.

The second endpoint category represented in Fig. 5, ecosystems quality, as opposed to human health, presents different trends when comparing laboratory and upscaled results. In the laboratory results, HMFO is the enzyme that contributes most to the category, reaching values of almost $9 \cdot 10^{-11}$ species·year; however, this is transposed in the upscaled scenario, with *Cgl*UPO being the enzyme that contributes most to the ecosystems quality damage category. The midpoint impact categories with most relevance to the endpoint category are, for all the analyzed scenarios, GW, TA and LU. In laboratory HMFO production, for instance, GW contributes with a 53% share, TA with 24% and LU with 8%. Other midpoint categories such as OF and FE also present a relevant contribution to the overall impact of laboratory HMFO (9 and 4%). For this endpoint category, the upscaled HMFO is the best-case scenario, with the lowest impact. When directly comparing the laboratory and their upscaled counterparts, *Cgl*UPO experiences a 46% reduction per functional unit and HMFO a 95% reduction.

With reference to the Monte Carlo uncertainty, the least data dispersion can be observed in the upscaled HMFO scenario ($4.03 \cdot 10^{-12} \pm 2.51 \cdot 10^{-12}$ species·year), following the same trend as in the previously discussed impact category. Data dispersion is quite relevant in this impact category, with quite large deviations from the mean. While HMFO shows less data dispersion when upscaled, this is not the case for *Cgl*UPO. Hereby, it can be concluded that the inventories for chemicals present larger

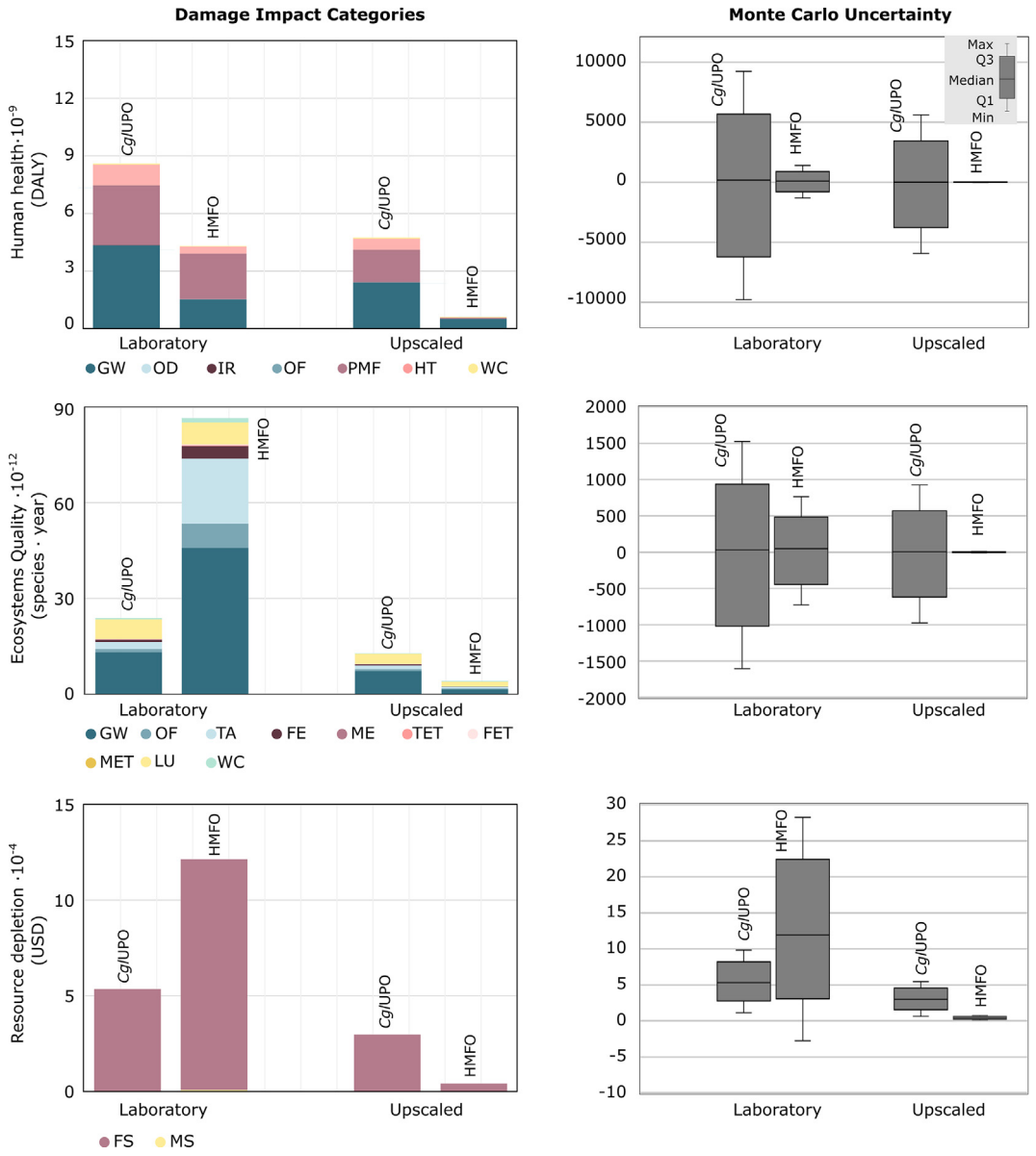

Fig. 5. Combined assessment of endpoint impact categories (human health, ecosystems quality and resource depletion) and the corresponding Monte Carlo uncertainty values for *Cgl*UPO and HMFO per functional unit.

uncertainties than that of the electricity mix. In the case of ecosystems quality, the greatest reductions in uncertainty are achieved when electricity consumption values are reduced through upscaling.

Regarding the last set of graphs in Fig. 5, fossil scarcity (FS) is the midpoint category primarily responsible for most impacts on the resource depletion endpoint indicator. Contrarily, metal scarcity (MS) does not present practically any contribution. This trend is accurate for every enzyme studied in the assessment of both production volumes. In this case, the most unfavorable production system is that of HMFO in laboratory scale. However, the results fluctuate for the upscaled systems, where *Cgl*UPO becomes the worst case with the highest contribution to the resource depletion category. In relative terms, when upscaled, *Cgl*UPO and HMFO experience a decrease of 44% and 97%, being HMFO the enzyme with the greatest improvement.

In terms of uncertainty, resource depletion is the endpoint impact category with the least data dispersion, resulting in the lowest standard deviation relatively. This effect may be due to the fact that resource depletion is affected by only two midpoint categories, FS and MS, while the other endpoint indicators have implications derived in a wider range of midpoint categories. The list of substances that globally contribute to the impact in these two categories will therefore be smaller than that of the other two endpoint categories. This means that more elementary flows will be involved in the final results, which will derive the aggregation of the effect of the uncertainties considered in the analyzed inventories. The standard deviations are within or below the order of magnitude of the mean values displaying the most representative endpoint category. The uncertainty of the samples is decreased in all cases for the upscaled scenarios compared to the laboratory experiments. The dataset with the least uncertainty is that of HMFO for the upscaled scenario of production with standard deviation value of $4.13 \cdot 10^{-5} \pm 3.97 \cdot 10^{-6}$ USD while *Cgl*UPO achieves $1.98 \cdot 10^{-6} \pm 1.80 \cdot 10^{-7}$ USD. The uncertainty in the resource depletion category is mainly affected by activities such as electricity

consumption (fossil-based energy production). As for the categories previously described, the dispersion of data is substantially reduced when data are upscaled for the scenarios in which electricity consumption is the main hotspot (HMFO). The significance of uncertainty datasets may be increased if real data on several trials were used for the Monte Carlo Assessment. However, since the objective of this study is to compare the scenarios analyzed, the results achieved are considered valuable for this purpose solely.

3.3. HMFO evaluation and benchmark

In general, the greatest difference in impacts occurs in the case of HMFO, from lab to upscaled scenarios. For the production of this enzyme, in which the primary dataset inventory is fairly complete, it is interesting to analyze which stage contributes most to impacts when analyzing a generic bioprocessing flowsheet. Fig. 6 displays the impacts for three relevant midpoint indicators in the assessment. GW is one of the most relevant indicators nowadays to describe a system environmentally. OD and ME were selected because they display the categories in which the upscale results in the lowest reductions, which, in turn, are still quite meaningful. In the upscaled results, the inventories were implemented slightly different, considering electricity and heating demands as separate activities contributing to each stage (shaded grey area in the graphs). In the laboratory scale scenarios, electricity consumption is considered within each stage. The figure presents in the same column, the impacts of both laboratory and upscaled scenario, which allows to see their differences in value. It also represents the impacts per stage of production: pre-inoculum, inoculum, bioreactor, microfiltration, and ultrafiltration.

For the three studied categories, the most relevant stage, regardless of the impact category, is the bioreactor stage with 57%, 58% and 60% contributions for GW, OD and ME. The pre-inoculum and inoculum stages are the stages with the least environmental impact, reaching a maximum of 3% contribution to the overall impact for the evaluated categories. Apart from the stage dealing

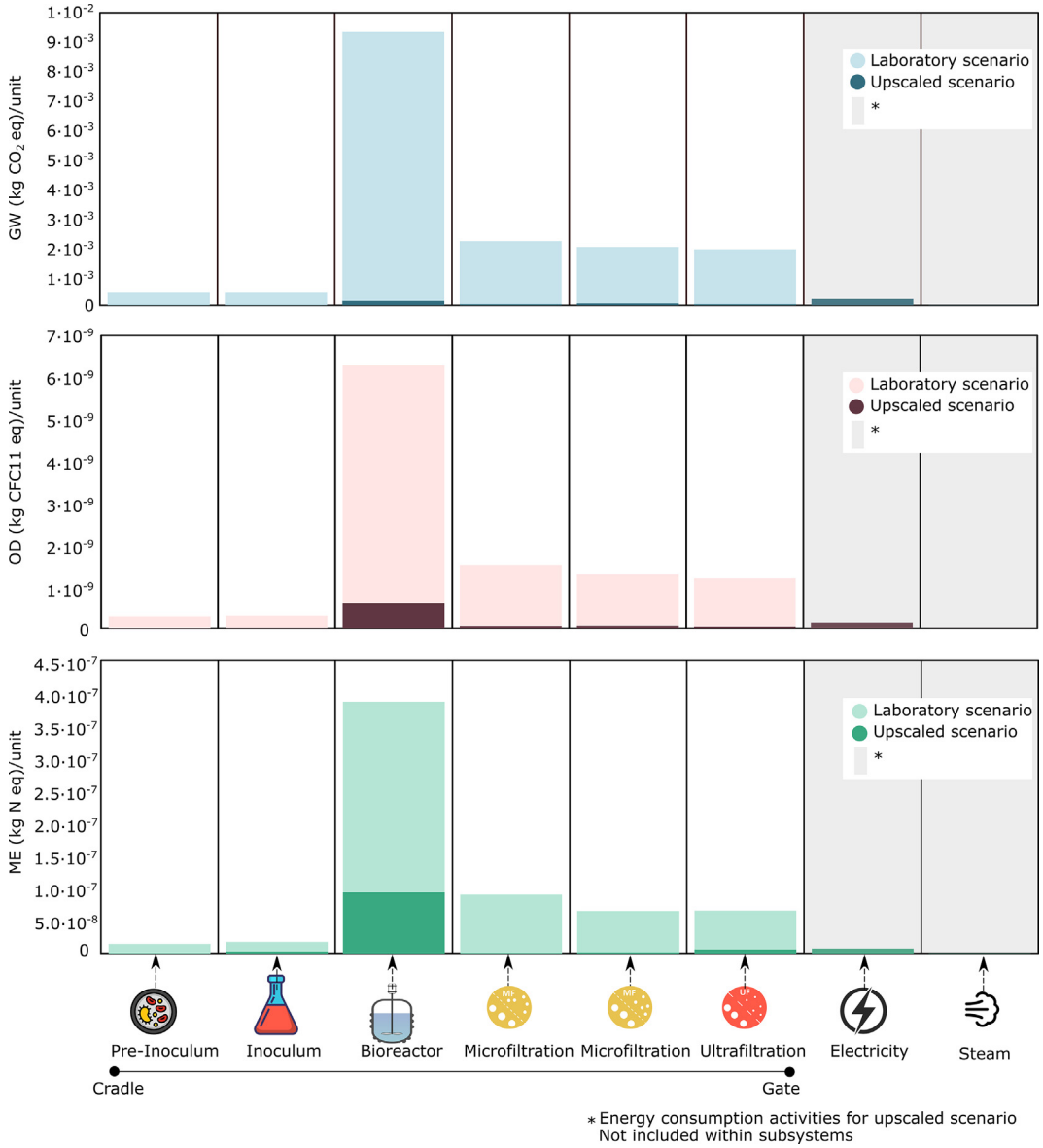

Fig. 6. GW: global warming, OD: ozone depletion and ME: marine eutrophication midpoint impact results displayed per processing subsystem and functional unit for the production of HMFO at laboratory scale and the upscaled scenario.

with the main fermenter, the downstream processing as a whole is a major contributing step, mainly due to the electricity consumption required for separation. The downstream stage, for the production of HMFO involves two subsequent microfiltration units, and an ultrafiltration step. While separately they present contributions about 10–14% each, if combined, the downstream is responsible for 34 and 37% of the overall environmental burden. The greatest contributor to the impacts in the laboratory scenario is electricity consumption, which is reduced in the upscaled version, where all the impact represented in each of the stages is due to other activities, mainly chemicals, nutrients and water.

While most studies present the results of the impact of enzymes with a functional unit based on mass-based values (1 kg enzyme), in this case it makes sense to analyze it as a function of units which will be useful in the sense of allowing future further implementation of LCA results within studies with extended system boundaries (e.g. enzymatic oxidation of HMF into FDCA).Whereas there are some published studies evaluating the environmental impact of certain enzymes, there are no studies specifically geared towards the assessment of enzymes directed to the enzymatic production of platform chemicals such as FDCA from lignocellulosic feedstock. There is no reported data involving an endpoint and uncertainty assessment.

The study of the environmental assessment of enzyme production is considered very relevant, since many studies have analyzed the significance of the environmental impacts of enzymes within a production route. For instance, Gilpin et al. (2017) present the attributional assessment of cellulase enzyme required for bioethanol production. In this case they analyze different case studies for the production of cellulase per kg of enzyme produced, concluding that the highest impact achieved is 10.6 kg CO_2 eq/kg enzyme. The great variation of results in the literature regarding the production of cellulases is attributed to the lack of a common framework for enzyme evaluation and the absence of adequate inventories. The GW result for HMFO production 98,729.13 kg CO_2/kg enzyme, is much higher, however, is not comparable to the results obtained in cellulase studies. Firstly, the objective or function of the enzymes is far from being the same, while the assumptions and scale of evaluations are completely different. The enzyme loads and activities are not depicted when mass-based systems are studied, which does not allow a fair comparison.

Although Delgove et al. (2019) present an LCA study of an enzymatic production process of functionalized lactones, using monooxygenases, they do not focus on the inventories and results obtained for the enzymes, but rather on the comparative assessment of enzymatic versus chemical routes of oxidation. However, some of the main conclusions reached in their study are similar to those attained in this assessment. Firstly, the authors express the relevance of the electricity consumption and electricity mix within laboratory scale processes. The GW impact of the enzymes represents a 16% contribution to the process, with a value of 0.26 kg CO_2 eq/g product. In this study, considering the use of 4000 units for the production of 15 g of FDCA, the GW impact for the HMFO enzyme produced in laboratory scale is 4.39 kg CO_2 eq/g FDCA, while if the upscaled experiment is considered, the impact is 0.14 kg CO_2/g FDCA. Regarding endpoint results, Delgove et al. (2019) report human health values of $1.64 \cdot 10^{-7}$ DALY/g product, while for HMFO with the assumed conditions for FDCA production the results would be $1.15 \cdot 10^{-5}$ DALY/g FDCA for the laboratory scenario and $4.32 \cdot 10^{-7}$ DALY/g FDCA for the upscaled scenario, attaining similar results to the baseline study. The authors also concluded that the Monte Carlo uncertainties acquired high values, and more for laboratory experiments than for industrial scale systems.

The results in this assessment indicate that the transformation of laboratory processes to upscale production is a requisite for the reduction of environmental impacts. Not only energy should be optimized, but also common laboratory procedures should be updated to more industrialized processes. For instance, the optimization of the fermentation mode of operation (e.g. continuous, fed batch) and medium composition could potentially increase productivity of the enzymes, thus reducing the impacts per functional unit. On the other hand, implementing sterilization processes in continuous mode, using steam, or optimizing the downstream separation sequence will potentially reduce, as well, the energy footprint of the system and maximize the final product yield. For example, cell disruption to release the enzymes has been performed through freezing, which increases the energetic consumption of the system. Other methods using chemicals could reduce the overall impact of the utilization of refrigeration chambers.

Other foreseeable improvements, at a broader scale, are in the way the utilization of enzymes is targeted. If enzymes were to be recovered and reutilized, their impacts would be reduced significantly. For instance, an option would be to recover them from the fermentation broth through the use of filtration membranes (Saha et al., 2017), the immobilization with hetero functional epoxy supports (Nath et al., 2014) or the immobilization and recovery through magnetic nanoparticles (Moldes-Diz et al., 2018). These options provide a way to potentially diminish the impact of enzyme utilization significantly. For instance, applying a hypothetical activity recovery after two cycles in the range of 31–100% (Saha et al., 2017), the reduction of the environmental impact of the use of enzymes could be reduced by 15.5–50%. After immobilization in epoxy-amino beads has shown the possibility of reutilizing β-Galactosidase without any loss in activity (Torres et al., 2003), which would suppose a 50% reduction in impacts if two the enzymes were to be reused for two cycles, and higher (i.e. environmental impact/number of cycles without activity loss) if more reuse cycles were achieved. Similarly, the use of immobilized laccase in silica magnetic nanoparticles for dye decolorization was implemented for 6 cycles, maintaining most of the activity of enzymes, which would mean a reduction of the environmental impact of about 6 times (Moldes-Diz et al., 2018).

4. Conclusions

This study was focused on filling the gap in issues related to environmental evaluation of the production of oxidative enzymes (HMFO and UPO) as support for the enzymatic transformation to obtain bioplastics. It was found that enzyme production through non-optimized, highly specialized low-volume production processes reveals electricity consumption as a major environmental hotspot. This hotspot shifts, in most cases, to the use of chemicals for the formulation of the culture medium when scale-up is performed. This study has confirmed that evaluating the environmental impacts of industrial enzymatic processes (large volumes of production) through data from laboratory scale experiments would incur in significant errors. Appropriate scale-up procedures are needed if environmental results for large production volumes are to be estimated from laboratory data. Laboratory-based LCA results may be valid as a predictive benchmark to set optimization objectives. According to the results, the laboratory scale processes always present a higher GW than their upscaled counterparts: 97 and 45% decrease for HMFO and *Cgl*UPO, due to the overestimated energy consumptions. The differences found in LCA results for enzymes with the same function, shows the need to include these and other biocatalysts within the scope and boundaries of environmental assessments of bio-based production systems. Further research should be focused on the development of databases with primary data on the production of various enzymes at different scales. The purpose is to be able to widen the knowledge on the real

environmental effect of substituting chemical production routes with bioprocessing enzymatic routes, aiming at a feasible increase in environmental sustainability of the obtained products.

CRediT authorship contribution statement

Sara Bello: Formal analysis, Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing, Visualization. **Noelia Pérez:** Investigation, Validation. **Jan Kiebist:** Investigation, Validation, Writing - review & editing. **Katrin Scheibner:** Validation, Supervision. **María Isabel Sánchez Ruiz:** Investigation, Validation. **Ana Serrano:** Investigation, Validation, Writing - review & editing. **Ángel T. Martínez:** Funding acquisition, Methodology, Supervision. **Gumersindo Feijoo:** Funding acquisition, Methodology, Supervision. **Maria Teresa Moreira:** Funding acquisition, Methodology, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was supported by the European EnzOx2 BBI JU-Project (H2020-BBI-PPP-2015-2-1-720297). S. Bello, G. Feijoo and M.T. Moreira belong to the Galician Competitive Research Group GRC ED431C 2017/29 and to the CRETUS Strategic Partnership (ED431E 2018/01). All these programmes are co-funded by FEDER (EU).

References

Aguilar, A., Twardowski, T., Wohlgemuth, R., 2019. Bioeconomy for sustainable development. Biotechnol. J. 14, 1–11. https://doi.org/10.1002/biot.201800638.

Albonetti, S., Lolli, A., Morandi, V., Migliori, A., Lucarelli, C., Cavani, F., 2015. Conversion of 5-hydroxymethylfurfural to 2 , 5-furandicarboxylic acid over Au-based catalysts : optimization of active phase and metal – support interaction. Appl. Catal. B Environ. 163, 520–530. https://doi.org/10.1016/j.apcatb.2014.08.026.

Álvarez, C., Reyes-Sosa, F.M., Díez, B., 2016. Enzymatic hydrolysis of biomass from wood. Microb. Biotechnol. 9, 149–156. https://doi.org/10.1111/1751-7915.12346.

Bourguignon, J., Sion, M.-X., Moreau, M., 1980. United States Patent 4233245. Preparation of Tris(Hydroxymethyl)aminomethane.

Carlsson, U., Ferskgard, P., Svensson, S.C.T., 1991. A simple and efficient synthesis of the inducer IPTG made for inexpensive heterologous protein production using the lac-promoter. Protein Eng. 4, 1019–1020.

Carro, J., Fernández-Fueyo, E., Fernández-Alonso, C., Cañada, J., Ullrich, R., Hofrichter, M., Alcalde, M., Ferreira, P., Martínez, A.T., 2018. Self-sustained enzymatic cascade for the production of 2, 5-furandicarboxylic acid from 5-methoxymethylfurfural. Biotechnol. Biofuels 11, 86–96. https://doi.org/10.1186/s13068-018-1091-2.

Carro, J., Ferreira, P., Rodríguez, L., Prieto, A., Serrano, A., Balcells, B., Ardá, A., Jiménez-Barbero, J., Gutiérrez, A., Ullrich, R., Hofrichter, M., Martínez, A.T., 2015. 5-Hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. FEBS J. 282, 3218–3229. https://doi.org/10.1111/febs.13177.

Carvalho, F.S., Fornasier, F., Leitão, J.O.M., Moraes, J.A.R., Schneider, R.C.S., 2019. Life cycle assessment of biodiesel production from solaris seed tobacco. J. Clean. Prod. 230, 1085–1095. https://doi.org/10.1016/j.jclepro.2019.05.177.

Cheng, M., Rosentrater, K.A., Sekhon, J., Wang, T., Jung, S., Johnson, L.A., 2019. Economic feasibility of soybean oil production by enzyme-assisted aqueous extraction processing. Food Bioprocess Technol. 12, 539–550.

Choudhury, A.K.R., 2020. Introduction to enzymes. In: Sustainable Technologies for Fashion and Textiles. Elsevier Ltd, pp. 75–90. https://doi.org/10.1016/B978-0-08-102867-4.00004-9.

Delgove, M.A.F., Laurent, A.B., Woodley, J.M., De Wildeman, S.M.A., Bernaerts, K.V., van der Meer, Y., 2019. A prospective life cycle assessment (LCA) of monomer synthesis: comparison of biocatalytic and oxidative chemistry. ChemSusChem 12, 1349–1360. https://doi.org/10.1002/cssc.201900007.

Dijkman, W.P., Fraaije, M.W., 2014. Discovery and characterization of a 5-hydroxymethylfurfural oxidase from Methylovorus sp. strain MP688. Appl. Environ. Microbiol. 80, 1082–1090. https://doi.org/10.1128/AEM.03740-13.

Domínguez de María, P., Guajardo, N., 2017. Biocatalytic valorization of furans: opportunities for inherently unstable substrates. ChemSusChem 10, 4123–4134. https://doi.org/10.1002/cssc.201701583.

Gavankar, S., Suh, S., Keller, A.A., 2014. The Role of Scale and Technology Maturity in Life Cycle Assessment of Emerging Technologies A Case Study on Carbon Nanotubes, vol. 19, pp. 51–60. https://doi.org/10.1111/jiec.12175.

Ghosh, A., Vijay Anand, K.G., Seth, A., 2015. Life cycle impact assessment of seaweed based biostimulant production from onshore cultivated Kappaphycus alvarezii (Doty) Doty ex Silva-Is it environmentally sustainable? Algal Res 12, 513–521. https://doi.org/10.1016/j.algal.2015.10.015.

Gilpin, G.S., Andrae, A.S.G., 2017. Comparative attributional life cycle assessment of European cellulase enzyme production for use in second-generation lignocellulosic bioethanol production. Int. J. Life Cycle Assess. 22, 1034–1053.

Goedkoop, M., Heijungs, R., Huijbregts, M., Schryver, A. De, Struijs, J., Zelm, R. Van, 2009. ReCiPe 2008, A Life Cycle Impact Assessment Method Which Comprises Harmonised Category Indicators at the Midpoint and the Endpoint Level. University of Leiden, Radboud University Nijmegen, RIVM, Bilthoven, Amersfoort, Netherlands.

Harding, K., 2008. A Generic Approach to Environmental Assessment of Microbial Bioprocesses through Life Cycle Assessment by.

Hong, Y., Nizami, A.-S., Bafrani, M.P., Saville, B.A., 2013. Impact of cellulase production on environmental and fi nancial metrics for lignocellulosic ethanol Yan. Biofuels, Bioprod. Bioref. 303–313. https://doi.org/10.1002/bbb.

Huang, Y.-T., Wong, J.-J., Huang, C.-J., Li, C.-L., Jang, G.-W.B., 2016. 2,5-Furandicarboxylic acid synthesis and use. In: Chemicals and Fuels from Bio-Based Building Blocks, pp. 191–215. https://doi.org/10.1002/9783527698202.ch8.

Huijbregts, M.A.J., Steinmann, Z.J.N., Elshout, P.M.F., Stam, G., Verones, F., Vieira, M.D.M., Hollander, A., Van Zelm, R., 2016. ReCiPe 2016: A Harmonized Life Cycle Impact Assessment Method at Midpoint and Endpoint Level. Bilthoven, The Netherlands.

ISO 14040, 2006. Environmental Management — Life Cycle Assessment — Principles and Framework.

ISO 14044, 2006. Environmental Management — Life Cycle Assessment — Requirements and Guidelines.

Isola, C., Sieverding, H.L., Raghunathan, R., Sibi, M.P., Webster, D.C., Sivaguru, J., Stone, J.J., 2017. Life cycle assessment of photodegradable polymeric material derived from renewable bioresources. J. Clean. Prod. 142, 2935–2944. https://doi.org/10.1016/j.jclepro.2016.10.177.

Karich, A., Kleeberg, S.B., Ullrich, R., Hofrichter, M., 2018. Enzymatic preparation of 2,5-furandicarboxylic acid (FDCA)—a substitute of terephthalic acid—by the joined action of three fungal enzymes. Microorganisms 6, 5. https://doi.org/10.3390/microorganisms6010005.

Katakojwala, R., Mohan, S.V., 2021. A critical view on the environmental sustainability of biorefinery systems. Curr. Opin. Green Sustain. Chem. 27, 100392 https://doi.org/10.1016/j.cogsc.2020.100392.

Kiebist, J., Schmidtke, K.U., Zimmermann, J., Kellner, H., Jehmlich, N., Ullrich, R., Zänder, D., Hofrichter, M., Scheibner, K., 2017. A peroxygenase from Chaetomium globosum catalyzes the selective oxygenation of testosterone. Chembiochem 18, 563–569. https://doi.org/10.1002/cbic.201600677.

Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B.A., Blanch, H.W., 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol. Bioeng. 109, 1083–1087. https://doi.org/10.1002/bit.24370.

Maclean, H.L., Spatari, S., 2009. The contribution of enzymes and process chemicals to the life cycle of ethanol. Environ. Res. Lett. 4 https://doi.org/10.1088/1748-9326/4/1/014001.

Martínez, Á.T., Ruiz-Dueñas, F.J., Martínez, M.J., del Río, J.C., Gutiérrez, A., 2009. Enzymatic delignification of plant cell wall: from nature to mill. Curr. Opin. Biotechnol. 20, 348–357. https://doi.org/10.1016/j.copbio.2009.05.002.

Moldes-Diz, Y., Eibes, G., Vázquez-Vázquez, C., Fondado, A., Mira, J., Feijoo, G., Lema, J.M., Teresa, M., 2018. A novel enzyme catalysis reactor based on superparamagnetic nanoparticles for biotechnological applications. J. Environ. Chem. Eng. 6, 5950–5960. https://doi.org/10.1016/j.jece.2018.09.014.

Nath, A., Mondal, S., Chakraborty, S., Bhattacharjee, C., Chowdhury, R., 2014. Production, purification, characterization, immobilization and application of β-galactosidase: a review. Asia Pac. J. Chem. Eng. 9, 330–348. https://doi.org/10.1002/apj.

Nielsen, P.H., Oxenbøll, K.M., Wenzel, H., 2007. Cradle-to-Gate Environmental Assessment of Enzyme Products Produced Industrially in Denmark by Novozymes A/S, vol. 12, pp. 432–438.

Olofsson, J., Barta, Z., Börjesson, P., Wallberg, O., 2017. Integrating enzyme fermentation in lignocellulosic ethanol production: life-cycle assessment and techno-economic analysis. Biotechnol. Biofuels 10, 1–14. https://doi.org/10.1186/s13068-017-0733-0.

Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T., Vandenberghe, L.P.S., Mohan, R., 2000. Biotechnological potential of agro-industrial residues . II : cassava bagasse. Bioresour. Technol. 74, 81–87.

Parajuli, R., Dalgaard, T., Jørgensen, U., Adamsen, A.P.S., Knudsen, M.T., Birkved, M., Gylling, M., Schjørring, J.K., 2015. Biorefining in the prevailing energy and materials crisis: a review of sustainable pathways for biorefinery value chains and sustainability assessment methodologies. Renew. Sustain. Energy Rev. 43, 244–263. https://doi.org/10.1016/j.rser.2014.11.041.

Parvatker, A.G., Eckelman, M.J., 2019. Comparative evaluation of chemical life cycle inventory generation methods and implications for life cycle assessment results. ACS Sustain. Chem. Eng. 7, 350–367. https://doi.org/10.1021/

acssuschemeng.8b03656.
Penman, J., Gytarsky, M., Hiraishi, T., Irving, W., Krug, T., 2006. 2006 IPCC - Guidelines for National Greenhouse Gas Inventories, Directrices para los inventarios nacionales GEI.
Piccinno, F., Hischier, R., Seeger, S., Som, C., 2018. Predicting the environmental impact of a future nanocellulose production at industrial scale : application of the life cycle assessment scale-up framework. J. Clean. Prod. 174, 283–295. https://doi.org/10.1016/j.jclepro.2017.10.226.
Raman, K., Henning, P.H., 2013. Environmental assessment of enzyme use in industrial production- a literature review. J. Clean. Prod. 42, 228–240. https://doi.org/10.1016/j.jclepro.2012.11.005.
Saha, K., R, U.M., Sikder, J., Chakraborty, S., da Silva, S.S., dos Santos, J.C., 2017. Membranes as a tool to support biorefineries: applications in enzymatic hydrolysis, fermentation and dehydration for bioethanol production. Renew. Sustain. Energy Rev. 74, 873–890. https://doi.org/10.1016/j.rser.2017.03.015.
Sajid, M., Zhao, X., Liu, D., 2018. Production of 2,5-furandicarboxylic acid (FDCA) from 5-hydroxymethylfurfural (HMF): recent progress focusing on the chemical-catalytic routes. Green Chem. 20, 5427. https://doi.org/10.1039/c8gc02680g.
Serrano, A., Calviño, E., Carro, J., Sánchez-Ruiz, M.I., Cañada, F.J., Martínez, A.T., 2019. Complete oxidation of hydroxymethylfurfural to furandicarboxylic acid by aryl-alcohol oxidase. Biotechnol. Biofuels 12, 1–12. https://doi.org/10.1186/s13068-019-1555-z.
Tong, X., Ma, Y., Li, Y., 2010. Biomass into chemicals: conversion of sugars to furan derivatives by catalytic processes. Appl. Catal. Gen. 385, 1–13. https://doi.org/10.1016/j.apcata.2010.06.049.
Torres, R., Mateo, C., Fernández-Lorente, G., Ortiz, C., Fuentes, M., Palomo, J.M., Guisan, J.M., Fernández-Lafuente, R., 2003. A novel heterofunctional epoxy-amino sepabeads for a new enzyme immobilization protocol: immobilization-stabilization of β-galactosidase from Aspergillus oryzae. Biotechnol. Prog. 19, 1056–1060. https://doi.org/10.1021/bp025771g.
Tranberg, B., Corradi, O., Lajoie, B., Gibon, T., Staffell, I., Andresen, G.B., 2019. Real-time carbon accounting method for the European electricity markets. Energy Strateg. Rev. 26 https://doi.org/10.1016/j.esr.2019.100367.
Triebl, C., Nikolakis, V., Ierapetritou, M., 2013. Simulation and economic analysis of 5-hydroxymethylfurfural conversion to 2,5-furandicarboxylic acid. Comput. Chem. Eng. 52, 26–34. https://doi.org/10.1016/j.compchemeng.2012.12.005.
Van Putten, R.J., Soetedjo, J.N.M., Pidko, E.A., Van Der Waal, J.C., Hensen, E.J.M., De Jong, E., Heeres, H.J., 2013. Dehydration of different ketoses and aldoses to 5-hydroxymethylfurfural. ChemSusChem 6, 1681–1687. https://doi.org/10.1002/cssc.201300345.
Viñambres, M., Espada, M., Martínez, A.T., Serrano, A., 2020. Screening and evaluation of new hydroxymethylfurfural oxidases for furandicarboxylic acid production. Appl. Environ. Microbiol. 86, 1–17. https://doi.org/10.1128/AEM.00842-20.
Wernet, G., Bauer, C., Steubing, B., Reinhard, J., Moreno-Ruiz, E., Weidema, B., 2016. The ecoinvent database version 3 (part I): overview and methodology. Int. J. Life Cycle Assess. 21, 1218–1230. https://doi.org/10.1007/s11367-016-1087-8.
Yuan, H., Liu, H., Du, J., Liu, K., Wang, T., Liu, L., 2020. Biocatalytic production of 2,5-furandicarboxylic acid: recent advances and future perspectives. Appl. Microbiol. Biotechnol. 104, 527–543. https://doi.org/10.1007/s00253-019-10272-9.