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A life cycle assessment of early-stage enzyme manufacturing simulations from sustainable feedstocks

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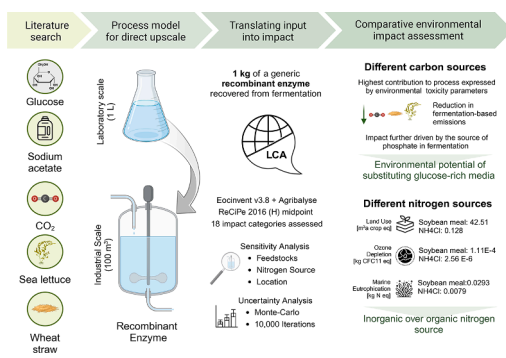
HIGHLIGHTS

- Six different enzyme production processes were designed and subjected to LCA.
- In all cases, human and ecosystem toxicity categories dominated the overall impact.
- Replacing glucose with CO_2, straw or *Ulva* greatly reduced fermentation emissions.
- Inorganic N sources had significantly lower impact then their organic counterparts.
- Electricity source of the country is a significant factor for the carbon footprint.

GRAPHICAL ABSTRACT



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ABSTRACT

Enzyme-catalyzed reactions have relatively small environmental footprints. However, enzyme manufacturing significantly impacts the environment through dependence on traditional feedstocks. With the objective of determining the environmental impacts of enzyme production, the sustainability potential of six cradle-to-gate enzyme manufacturing systems focusing on glucose, sea lettuce, acetate, straw, and phototrophic growth, was thoroughly evaluated. Human and ecosystem toxicity categories dominated the overall impacts. Sea lettuce, straw, or phototrophic growth reduces fermentation-based emissions by 51.0, 63.7, and 79.7%, respectively. Substituting glucose-rich media demonstrated great potential to reduce marine eutrophication, land use, and ozone depletion. Replacing organic nitrogen sources with inorganic ones could further lower these impacts. Location-specific differences in electricity result in a 14% and a 27% reduction in the carbon footprint for operation in Denmark compared to the US and China. Low-impact feedstocks can be competitive if they manage to achieve substrate utilization rates and productivity levels of conventional enzyme production processes.

1. Introduction

Enzymatic catalysis offers a valuable contribution towards the United Nations' Sustainable Development Goals (SDGs) clean energy (SDG 9), climate action (SDG 13), life below water (SDG 14) and life on land (SDG 15), thereby expediting social objectives, such as no poverty (SDG 1), zero hunger (SDG 2) and good health (SDG 3). A comprehensive study indicates that the environmental impacts of conventional

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industrial processes can be greatly reduced by implementing enzymatic processes instead (Jegannathan and Nielsen, 2013). Nevertheless, these assessments frequently lack comprehensive data concerning the influence of different enzyme production pathways on the overall biotransformation process. Consequently, authors often heavily depend on databases, which may overlook differences in secretion mode, purity, and media components. Considering the United Nations SDGs emphasizing responsible manufacturing practices (SDG 12), there is a pressing need to confront the challenge of identifying and quantifying the key environmental determinants of different enzyme production strategies. Life cycle assessments (LCA), a methodology to assess the environmental sustainability of a product throughout its life cycle (European Commission, 2010) should be considered, even in early development of production technology.

Manufacturing conditions such as carbohydrate utilization, fermentation time, formulation type, yield, and final enzyme activity give rise to large variations in environmental impacts (Nielsen et al., 2007). An early-stage assessment of two oxidative enzymes identified fermentation media to be the main source of marine eutrophication, land use, ozone depletion, and climate change. Within these categories, refined glucose, soybean meal (SBM), and other culture medium chemicals accounted for up to 75 % of all impact categories evaluated (Bello et al., 2021). Furthermore, in an example of industrial-scale production of intracellular β-galactosidase, the downstream processing unit, in particular chemicals used for enzyme purification, had significant contributions to the environmental impact of the process (Feijoo et al., 2017). An LCA on direct production data performed by Novozymes® emphasized that genetically modified microorganisms hold the potential of up to a 6-fold decrease in climate change, acidification, nutrient enrichment, and ozone formation from enzyme production compared to wild-type microorganisms (Nielsen et al., 2007). Although there are several other somewhat related studies in the literature, their scope is limited to the utilization, but not the manufacturing of fungal cellulases in bioethanol production, with a focus on greenhouse gas emissions, ignoring other environmental impact categories, with the exception of (Gilpin and Andrae, 2017), who assessed three alternative carbon sources for fungal cellulase production in Europe, and reported that in all scenarios, carbon source was the major contributor across nearly all the impact categories investigated.

In this study, with the objective to assess the environmental performances of different recombinant enzyme (rEnz) manufacturing strategies, six recombinant enzyme production routes with various host organisms and feedstocks were simulated, namely *Bacillus licheniformis* on glucose (Bl_Glc, reference process), *B. licheniformis* on *Ulva fenestrata* biomass (Bl_Ulv), *Escherichia coli* on glucose (Ec_Glc), *Lysinibacillus sphaericus* on acetate (Ls_Ace), *Aspergillus oryzae* on wheat straw (Ao_Str), and *Synechococcus elongatus* on CO_2 (Se_CO_2) and their environmental impacts compared. With excellent secretory pathways in place, enzyme production in *B. licheniformis* growing on defined glucose media is already commercially applied (Oesterling and Affairs, 2020) and is used here as the reference process. *E. coli*, an intracellular enzyme expression host, is frequently used for protein research and therapeutics production (Ferrer-Miralles et al., 2009). Therefore *E. coli* growing on defined glucose media was also considered (Ferreira et al., 2018). Fermentation media, in particular, using refined glucose as the carbon source, have been shown to strongly contribute to the environmental profile of the overall enzyme production line (Bello et al., 2021). Fermentation on highly abundant wheat straw, therefore, appears to hold the potential to diminish the impacts of substrate manufacturing. The ability of the fungus *A. oryzae* to produce recombinant enzymes when growing on straw in solid-state-fermentation (SSF) (Shinkawa and Mitsuzawa, 2020) was thus also explored in this study. To counteract the large amounts of arable land use in correspondence to fermentation ingredients, the marine macroalga *Ulva fenestrata* (sea lettuce), which was proven to produce high-quality biomass even under variable natural culturing conditions (Kidgell et al., 2019) was also investigated as a feedstock. In a recent study, researchers have successfully engineered *B. licheniformis* to enable utilization of the cell-wall bound algal heteropolysaccharide, ulvan, as the sole carbon source (Dutschei et al., 2022), providing the fundament for an LCA. The third substrate of interest is acetate since C1 and C2 compounds are generally believed to have enormous potential for sustainability (due to production routes from waste streams or direct utilization of CO_2.). *L. sphaericus*, a mesophilic, halotolerant soil bacterium, can grow on mineral media with acetate as the sole carbon source. The first approaches of the successful transformation of foreign DNA (Fu et al., 2017) provide a fundament for *L. sphaericus* to potentially function as a rEnz expression host. Lastly, capturing CO_2 directly, and transforming it into biomass, appears to have the most promising effect on mitigating climate change. Cyanobacteria, such as *S. elongatus,* present an interesting biotechnology platform through efficient phototrophic growth, low nutrient requirements, and tolerance to high salt, temperature, or light. With its natural genetic transformability, it is well-suited as another rEnz producer (Jaiswal et al., 2020).

The diverse applications of enzymes present a considerable challenge when conducting environmental assessments of entire end-consumer processes. Given that enzyme production is presumed to represent the most significant component of environmental impact in biotransformation, the perspective of alternative carbon and nitrogen sources utilized in previously described fermentation systems of low technology readiness levels was under investigation here. Indeed, previous studies in the field simply lack this comprehensive approach to compare the impacts of different potentially low-impact feedstocks, organic and inorganic nitrogen sources, as well as host organisms. Location-specific impacts were also identified through an additional sensitivity analysis in this study. To the best of our knowledge, this is the most comprehensive work in the literature yet regarding the environmental impact assessment of enzyme manufacturing since six markedly different process simulations on various C and N sources were elucidated along with location-specific sensitivity analyses. The results presented here provide novel insight into environmental impacts of different rEnz production strategies with special emphasis on feedstocks and host organisms that have been widely assumed to be sustainable in past studies and draws a compelling picture that an LCA approach is essential when designing bioprocesses (since major contributors like electricity can still be the main "bottlenecks" in the way of sustainable design).

2. Methods

2.1. Goal and scope

The environmental performance of different rEnz production systems was assessed with an attributional LCA under ISO Standards 14044 (International Organization for Standardization (ISO), 2006) and the ILCD Handbook (European Commission, 2010). The environmental profile was assessed on the grounds of an attributional LCA. The goal of this study was the comparative assertion of the overall environmental impacts of six different rEnz production processes in Denmark. The functional unit was defined as one kg of rEnz recovered from batch fermentation. This analysis provides a starting point for the comprehensive evaluation of early-stage manufacturing processes for a universal rEnz.

2.2. System boundary

The production system follows a standard sequence of substrate pretreatment, three-stage seed fermentation, followed by product fermentation, and downstream operations. A generic cradle-to-gate system is represented in Fig. 1. The strain design processes for the development of the rEnz expression hosts were not considered within the scope of this work.

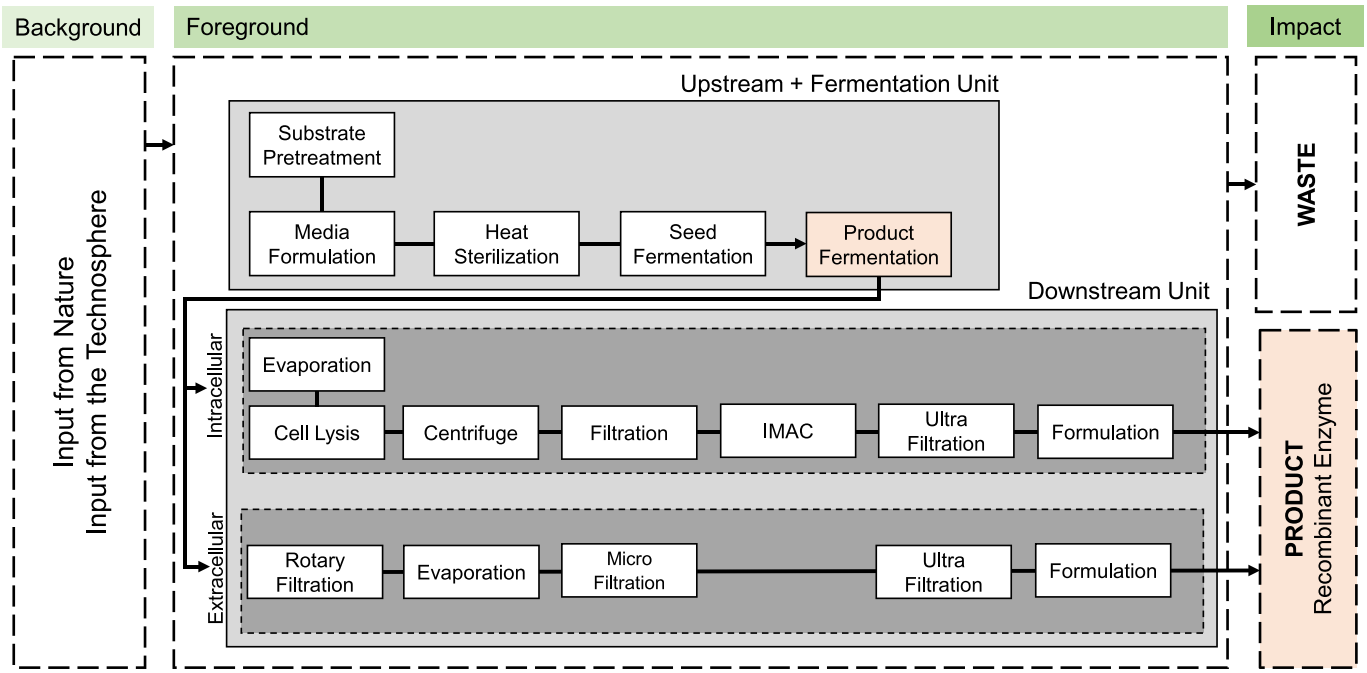

Fig. 1. Cradle-to-gate system boundary of the production of generic recombinant enzymes.

2.3. Life cycle inventory

The study was conducted as a preliminary LCA based on literature values of different technology-readiness levels. The evaluated processes were designed using SuperPro® Designer v13.1 (Intelligen, US). Calculations were performed on openLCA 1.10.3 using the ecoinvent v3.8 database as the source of background systems. Data entries were selected under the system cut-off. The complete inventory can be found in Table 1.

2.3.1. Upstream unit

Seed fermentation was upscaled by a factor of 100, going from 0.1 dm^3, 10 dm^3, and 1,000 dm^3 with an inoculum volume of 1 % (v/v) to the final product fermentation reaction. Before SSF, wheat straw underwent an additional two-stage pretreatment process based on ammonia, and enzymatic hydrolysis (Humbird et al., 2011; Shinkawa and Mitsuzawa, 2020) for better utilization of fermentable sugars. From a composition of 36.8 % glucan, 25.8 % xylan or other C5 sugars, and 15.8 % lignin (Nguyen et al., 2010), conversion rates were derived from Humbird et al. (2011).

2.3.2. Fermentation unit

Simulations were directly scaled up to a reaction volume of 100 m^3. The total input of proposed carbon sources was estimated at 10,000 kg, built on different high-cell density production models (Ferreira et al., 2018; Khatun et al., 2021; Niu et al., 2009). Based on the calculated stoichiometric ratios with a biomass yield of 0.5 g g^{-1} (Korz et al., 1995), the basis of mass balance was to utilize 50 % of the carbon, in excess of nitrogen, which is 2,500 kg of NH_4Cl. Other media ingredients, such as salts and growth supplements were added separately. Biomass production was simulated on the molar mass balances considering an empirical formula of $CH_{1.8}O_{0.5}N_{0.2}$ for heterotrophic organisms (Ferreira et al., 2018), $CH_{1.72}O_{0.52}N_{0.17}$ for fungal biomass (Carlsen et al., 1996), and $CH_{1.59}O_{0.27}N_{0.19}$ for S. elongatus (Shastri and Morgan, 2005). For extracellular secreted rEnz, values of 10 % of total biomass content were presumed (Çalik et al., 2003; Niu et al., 2009).

2.3.3. Downstream unit

The organization of extracellular rEnz purification has been modified from the Novozymes® production baseline (Oesterling and Affairs, 2020). After fermentation, rotary vacuum filtration is applied, as the primary separation step, to remove biomass and other impurities from the fermentation broth. Followed by concentration using evaporation, the mixture is filtered through a 0.5 μm mesh to completely remove any remaining residual strains. The rEnz product is stored in a solution of 5–10 % sodium chloride, 5–10 % sucrose, 1 % sodium benzoate and 1 % potassium sorbate – with a final concentration of 12.5 % (Oesterling and Affairs, 2021).

In cases of intracellular protein targeting, the biomass should be separated prior to rEnz purification. To allow for improved, cost-effective homogenization, high amounts of water are removed from the fermentation broth using flash evaporation. The host cells are then broken down and rEnz is released with a mass ratio of 0.1 g g^{-1} (Korz et al., 1995; Milo and Phillips, 2015). With a combination of centrifugation and dead-end-filtration, any remaining debris and biomass are removed. Through affinity chromatography, the target rEnz is then separated from other extracted proteins. After loading the sample mixture and eluting with a gradient of sodium chloride, the column is equilibrated, washed, and regenerated. While the column is treated with a series of tri(hydroxymethyl)-aminomethane (Tris) HCl, 0.1 M NaCl and 0.5 M NaOH washing, the eluent undergoes ultrafiltration to reduce the salt content and to concentrate the final product. The rEnz mixture is then preserved as described above (Oesterling and Affairs, 2020).

2.4. Assumptions and limitations

In order to allow for an evaluation of the sustainability potential, the scenarios are modelled with the same biomass and rEnz yields, neglecting the potential metabolic effects different rEnz expression strategies may have on the host organisms. The main assumptions were based on data availability. Materials, chemicals, or other services with no genuine record within the ecoinvent database were either replaced by closely related products or imported from the AGRIBALYSE® database. *Ulva* was modelled using AGRIBALYSE® data obtained from optimized sea lettuce production. Chilled water has been summarized under cooling water. Monopotassium phosphate was replaced with sodium phosphate. Inventory for Tris-HCl was built on stoichiometric ratios (Bourguignon et al., 1979), and the energy flow within the process

Table 1
Inventory summary for the 100 m^3 production of a generic recombinant enzyme.

Inputs for 1 kg recombinant enzyme		Bl_Glc	Bl_Ulv	Ls_Ace	Ao_Str	Ec_Glc	Se_CO_2
Seed Fermentation							
Media components							
Ammonium Chloride	kg	0.118	0.024	0.173	0.385	0.003	0.156
Carbon Source	kg	0.024	0.012	0.346	0.019	0.016	0.058
Phosphate Source	kg	0.021	0.021	0.034	0.004	0.028	0.0001
Salts	kg	0.001	0.012	0.001	0.002	0.002	0.0002
Others	kg	0.005	0.005	0.000	0.002	0.002	0.0003
Water	m^3	0.085	0.006	0.167	0.015	0.012	0.372
Utilities							
Cooling Water	m^3	0.037	0.000	0.006	0.158	0.000	0.000
Steam	kg	26.5	21.9	31.9	21.9	29.3	24.2
Electricity	kWh	0.187	0.143	0.375	1.055	0.189	0.407
Product Fermentation							
Media components							
Ammonium Chloride	kg	5.8	6.9	8.5	9.6	7.8	7.6
Carbon Source	kg	23.4	82.2	34.5	38.5*	31.0	31.1
Phosphate Source	kg	2.1	2.1	3.4		5.1	0.00012
Salts	kg	0.125	0.124	0.525			0.011
Others	kg	0.028	0.028	0.016	0.011	0.683	0.025
Water	m^3	0.205	0.304	0.297	0.000	0.446	0.299
Utilities							
Cooling Water	m^3	0.156	0.387	0.152	0.155	0.170	0.465
Electricity	kWh	66.2	229.7	102.6	38.2	53.0	281.7
Downstream							
Purification							
Eluent	kg					7.5	7.6
Tris – HCl	kg					102.6	115.2
Sodium Hydroxide	kg					12.9	14.5
Water	m^3	0.047	0.043	0.057	0.116	3.455	5.986
Steam	kg	267.7	263.9	376.3	268.0	336.2	251.7
Cooling	kg	89.5	82.8	124.9	85.9	196.6	137.2
Electricity	kWh	0.031	0.334	0.056	0.166	1.066	0.957
Formulation							
Sucrose	kg	0.457	0.475	0.526	0.513	0.486	0.486
Sodium Benzoate	kg	0.074	0.077	0.086	0.084	0.079	0.079
Potassium Sorbate	kg	0.074	0.007	0.001	0.084	0.079	0.079
Sodium Chloride	kg	0.457	0.475	0.526	0.513	0.486	0.486
Output to Technosphere							
rEnz	kg	1	1	1	1	1	1
Emissions to air (CO_2 from fermentation)	kg	19.7	10.0	14.8	12.8	19.7	0.4
Liquid Waste to WWTP	m^3	0.017	0.017	0.034	0.015	3.547	3.967
Biowaste to Incineration	kg	14.9	14.6	19.3	111.5	20.0	19.3
*Substrate Pretreatment							
Enzymes	kg				2.1		
Liquid Ammonia	kg				9.6		

was not included. Life cycle inventory for sodium acetate production was derived from (Jungbluth and Nguyen, 2008). The Impact of wheat straw was generalized under the ecoinvent entry for straw. Furthermore, formulation chemicals such as sodium benzoate, sucrose and potassium sorbate were replaced with benzoic acid, glucose, and potassium carbonate, respectively. All baseline scenarios in the model were designed with ammonium chloride as the primary nitrogen source. This choice aligns with the common utilization of ammonium chloride in M9 media, a well-defined growth medium widely used for the cultivation of different bacterial and fungal species. To allow the subsequent replacement of ammonium chloride with SBM, the nitrogen demand for the process was calculated from the elemental composition of $C_{4.81}H_{9.49}O_{2.68}N_{1.28}$ (Humbird, 2021). The introduction of SBM is not assumed to alter any other process parameters. For location-specific impact analysis, region-explicit market data was only available for electricity and water. Further distinctions could be made globally, or on the European level. The data gap allowed only for the following entries to be distinguished between these two types of market categories: sodium phosphate, steam, benzoic acid, calcium chloride, straw, energy, and transport parameters for sodium acetate and *Ulva* production, wastewater, dichloromethane, formaldehyde, and methyl chloride used for Tris-HCl reaction. Emission to air, in the form of ammonia, was divided into data for Europe and unspecified data applied in the location analysis for China and the US. The impact from all other forms of entries originates from globally derived market data. Data uncertainty factors were derived from basic uncertainty metrics sourced from ecoinvent v3.8, and pedigree matrix indicators were expertly chosen. Subsequently, Monte Carlo sampling was conducted on midpoint characters using the respective openLCA software module, executing 10,000 iterations with lognormally distributed uncertainty parameters.

2.5. Life cycle impact assessment

The ReCiPe 2016 midpoint hierarchical approach was applied, and normalization was performed against the World 2010 (H) database. The selected methodology covers a broad range of impact categories including relevant ones (such as land use, climate change, water consumption, *etc.*) for bioprocess development. In addition, this methodology has the advantage of providing impacts at both midpoint and endpoint levels. The corresponding impact categories used for this analysis are described in Table 2.

Table 2
Life Cycle parameters for evaluation of 1 kg of a generic recombinant enzyme.

Impact Category	Abbreviation	Unit
Fine particulate matter formation	FPMF	kg PM2.5 eq
Fossil resource scarcity	FRS	kg oil eq
Freshwater ecotoxicity	FET	kg 1,4-DCB
Freshwater eutrophication	FE	kg P eq
Global warming	GW	kg CO_2 eq
Human carcinogenic toxicity	HCT	kg 1,4-DCB
Human non-carcinogenic toxicity	HNCT	kg 1,4-DCB
Ionizing radiation	IR	kBq Co-60 eq
Land use	LU	m^2a crop eq
Marine ecotoxicity	MET	kg 1,4-DCB
Marine eutrophication	ME	kg N eq
Mineral resource scarcity	MRS	kg Cu eq
Ozone formation, Human health	OFH	kg NO_x eq
Ozone formation, Terrestrial ecosystems	OFT	kg NO_x eq
Stratospheric ozone depletion	OD	kg CFC11 eq
Terrestrial acidification	TA	kg SO_2 eq
Terrestrial ecotoxicity	TET	kg 1,4-DCB
Water consumption	WC	m^3

3. Results and discussion

3.1. Environmental impacts of rEnz production on different carbon sources

In this work a combined process design was employed, as well as an LCA workflow based on literature data to assess the environmental performances of six different feedstocks as carbon sources and ammonium chloride as low-impact nitrogen alternative for both extracellular (four processes) and intracellular (two processes) rEnz manufacturing in five different host organisms. According to the process simulations, extracellularly produced enzymes can reach a final amount of 423 and 426 kg/batch of fermentation medium for the Bl_Glc and Bl_Ulv processes, respectively, while extracellular enzyme manufacturing with Ls_Ace and Ao_Str process simulations could produce only 287 and 260 kg/batch, respectively, resulting from the molar-based bioconversion and wheat straw's limiting availability of fermentable sugars. With an additional extraction and purification step due to intracellular expression, a final amount of 322 and 321 kg/batch enzyme was achieved in Ec_Glc and Se_CO_2 processes, respectively. The impacts on environmental categories of each scenario are presented in Table 3. The reference scenario Bl_Glc exhibited the lowest emission in 8 out of 18 categories across all studied systems. In the case of Bl_Ulv, reduction in FPMF, LU, TA, and TET became evident, whereby emission towards ME and OD were lowered by 64.3 and 47.4 %, respectively. Fermentation using Ls_Ace could further decrease LU and ME factors by a factor of two. The drastic difference between these feedstocks and glucose fermentation, within these categories, seems to be consistent with the results of Bello et al. (2021). Previous research has indicated that the high OD values associated with glucose are primarily attributable to starch manufacturing and pretreatment operations (Blanco et al., 2020). Additionally, the application of ammonium fertilizers and agricultural land for crop culturing explains this severe divergence between categories like ME and LU. Interestingly, using wheat straw (Ao_Str) does not positively influence these factors. As outlined in (Dunlap et al., 2024), the GW in fermentation-based bio-succinic acid production is comparatively lower when utilizing corn starch (a glucose precursor) instead of employing seaweed or agricultural waste such as wood. This observed trend in GW was also evident in this study. Next to a 3.7 % reduction in MET, 5.5 % in TET, and 14.9 % in HCT, lower contributions towards FPMF, MRS, and TA are seen. For intracellular expression (Ec_Glc), following chromatography-based purification results in an increased impact on all categories when comparing to Bl_Glc. Notably, even direct carbon fixation (Se_CO_2) does not allow for intracellular expression to be environmentally competitive, except in the case of LU.

Each environmental flow was normalized against World 2010 (H) for evaluation of the most impactful system. Surprisingly, none of the

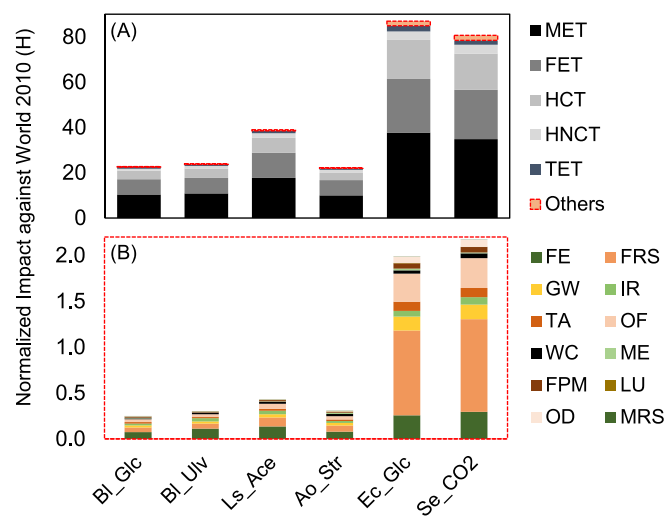

Fig. 2. A: Normalized environmental impacts of the six rEnz production scenarios., B: A detailed look at the boxed areas in panel A.

Table 3
Comparative evaluation of midpoint categories for ReCiPe 2106 (H) analysis of the six rEnz production strategies evaluated. Red: Impact > Bl_Glc; Yellow: Impact = Bl_Glc (±5%); Green: Impact < Bl_Glc.

Impact Category	Unit	Bl_Glc	Bl_Ulv	Ls_Ace	Ao_Str	Ec_Glc	Se_CO_2
FPMF	kg PM2.5 eq	0.2144	0.1879	0.3550	0.2122	1.4907	1.4835
FRS	kg oil eq	49.0686	52.5027	93.7699	66.0161	905.1429	989.9581
FET	kg 1,4-DCB	8.1910	8.4260	13.7079	8.1924	29.0390	26.7000
FE	kg P eq	0.0491	0.0737	0.0893	0.0518	0.1671	0.1921
GW	kg CO2 eq	176.9574	185.2487	284.6472	221.5846	1224.6302	1271.5664
HCT	kg 1,4-DCB	10.4399	11.4196	18.2833	8.8835	47.8405	44.3144
HNCT	kg 1,4-DCB	132.8002	181.3113	295.8154	197.4216	562.3607	576.1254
IR	kBq Co-60 eq	9.7382	16.9640	18.1123	10.2043	28.8854	38.4843
LU	m2a crop eq	21.5726	13.0134	9.5822	30.1955	32.6930	19.5552
MET	kg 1,4-DCB	10.7465	11.1637	18.2153	10.3504	38.9411	35.9993
ME	kg N eq	0.0406	0.0144	0.0199	0.0700	0.0842	0.0507
MRS	kg Cu eq	0.6707	0.5780	1.0560	0.5884	2.6450	2.1267
OFH	kg NOx eq	0.2797	0.3013	0.5122	0.3470	2.8566	3.0545
OFT	kg NOx eq	0.2853	0.3068	0.5268	0.3544	2.9741	3.1825
OD	kg CFC11 eq	1.89E-04	1.04E-04	1.88E-04	2.60E-04	4.13E-03	4.47E-03
TA	kg SO2 eq	0.6643	0.5319	0.8769	0.6539	4.1469	4.0319
TET	kg 1,4-DCB	769.4534	630.9106	1180.8671	726.9304	2584.5834	2082.0502
WC	m3	2.5558	3.9943	5.9211	6.0248	9.2976	13.5003

scenarios were found to be superior to others, including the baseline scenario, in terms of their overall environmental impacts (Fig. 2). Analysis of the normalized midpoint categories identified toxicity indicators HCT, HNCT, FET, MET, and TET to dominate all six rEnz production processes' environmental performances by almost 99 % of the overall impact (Fig. 2). Categories like LU, ME, OD, and GW, described in the literature to be severely affected by fermentation of chemicals (Bello et al., 2021), appear to be less significant in rEnz production.

3.2. Flow analysis of toxicity parameters

Fig. 2 elucidates that almost 99 % of the overall generated impact is allocated between different toxicity parameters. To understand the individual contributors within each category, a flow analysis was performed on the five toxicity categories: HCT, HNCT, TET, FET, and MET. In scenarios, Bl_Glc and Ls_Ace, the main fermentation step was responsible for almost half of all the chemical emissions normalized to the form of 1,4-DCB through the production of media components. Specifically, the carbon source alone accounted for 25.0 and 30.9 % of total toxicity impacts, respectively. Nielsen et al. (2007) also concluded that the fermentation step was the main responsible for the environmental impact of enzyme manufacturing due to electricity consumption and medium ingredients. Gilpin and Andrae (2017) assessed cellulase production on corn starch glucose, sugar cane molasses or pretreated softwood scenarios and reported similar results: For the impact categories eutrophication potential, acidification potential, photochemical oxidation potential, land use, and cumulative energy demand, carbon source was the main contributor. Overall, pretreated softwood was suggested as the carbon source with the lowest impact compared to the other two, however it caused almost a 10-fold and 80-fold increase in land use as compared to sugar cane molasses and corn starch glucose scenarios, respectively.

The toxicity level associated with glucose as primary feedstock in Bl_Glc has been correlated to starch production or pretreatment using glucose derived from maize or woody biomass (Blanco et al., 2020). Sodium acetate only gains an impact reduction in LU, ME and OD compared to glucose within the investigated categories. The observed effects of sodium acetate can be linked to the production of acetic acid and sodium hydroxide, which are fundamental building blocks in chemically synthesized sodium acetate, the main market dominator. Electrosynthesized acetate offers a means to circumvent the existing hotspots in production although high demand of electricity and low titres may impede the environmental benefits of direct CO_2 to product conversion. On the contrary, in scenarios Bl_Ulv and Ao_Str, the primary carbon source itself only expressed 2.8 % and 4.1 % impact, respectively. The limited resources necessary for harvest and preparation of sea lettuce directly impacts flows like marine toxicity from 0.09 kg 1,4-DCB compared to glucose of 2.7 kg 1,4-DCB. While wheat straw itself presents low toxicity (0.17 kg 1,4-DCB), including the necessary pretreatment for enhanced sugar availability, it increases the impact within this category to 2.8 kg 1,4-DCB, 8 % higher than that of glucose. The environmental hotspot of two-stage pretreatment is expressed by enzymatic hydrolysis, as highlighted by previous studies of different straw-to-product fermentations. Modifying the initial stage treatment, such as steam explosion (Rebolledo-Leiva et al., 2022), fails to meet lowered performance criteria, prompting a more accelerated exploration of low-resource approaches, or direct utilization of the substrate through engineered microorganisms. In the case of Bl_Ulv, electricity consumption, particularly for fermentation operations, constitutes the largest fraction within these environmental flows (Fig. 3). Lower productivity on this substrate, as compared to the reference example (3 mg $g^{-1}h^{-1}$ vs. 10.4 mg $g^{-1}h^{-1}$), requires the need for longer cultivation periods and thus compromises the otherwise environmentally competitive production. Engineering efforts aimed towards higher substrate throughput,

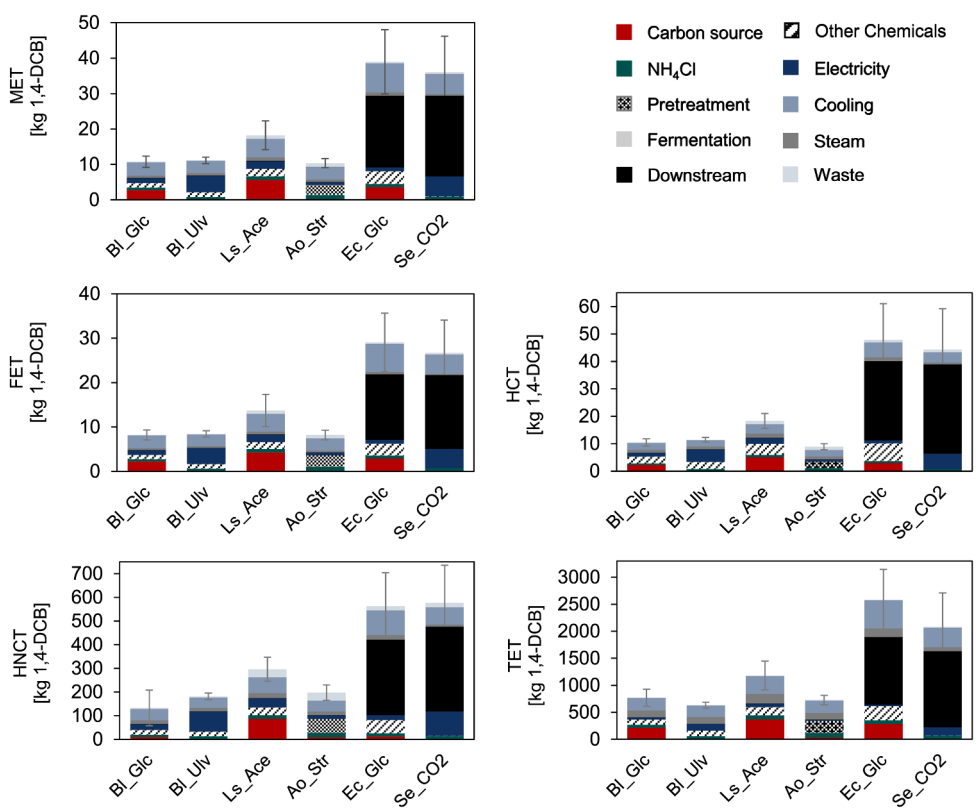

Fig. 3. Flow analysis for rEnz production of the most impacted midpoint categories (>accumulated 90 % cut-off) identified by normalization step (World 2010 (H)). Systems are divided based on the main carbon source used during fermentation. Error bars express standard deviations derived from Monte-Carlo simulations on 10,000 iterations.

consequently reducing electricity demand for fermentation operations, could allow an overall reduction in toxicity of up to 20.5 %.

Another source of toxicity arises from phosphate, which is employed both as a buffering agent and a source of phosphorus for microbial growth. In the respective reference scenario, levels of 12.1 % for MET, 24.5 % for HCT, and 14.9 % for TET alone, are illustrated within the chemical fraction in Fig. 3. The origin of these toxicity values is traced back to the phosphorus source itself, in particular wet beneficiation of rock phosphate (Smol et al., 2019). In scenarios Ao_Str and Se_CO_2, where either no – or small – amounts of phosphate sources were applied, a notable reduction of 65.5 and 79.9 % in impact generated by fermentation media was observed. Wheat straw being naturally abundant in phosphorus can be directly utilized by filamentous fungi (Shahryari et al., 2018), thus creating an environmental advantage over scenarios with additional phosphate supplementation. Furthermore, the toxicity impact from cooling accounted for up to 31.5 % of the total toxicity impact (Bl_Glc). The sodium chloride brine solution used for cooling is either extracted during salt mining or generated as a waste product of various chemical processes, where the presence of heavy metals or organic contaminants adds to the environmental complexity of the brine (Katal et al., 2020). Although undergoing different waste treatment methods, the complete removal of these constituents is not possible, which leads to ecosystem pollution and damage to human health (Backer et al., 2022; Panagopoulos et al., 2019). Another environmental hotspot is created from chromatography-based purification, in particular, washing agents such as Tris-HCl. In Ec_Glc and Se_CO_2, 1,4-DCB emissions increased significantly by 3.8 and 3.4-fold for MET, solely due to the implementation of this downstream operation. These significantly elevated toxicity impacts are primarily accountable for a 4.6-fold increase in the overall impact of these scenarios in reference to Bl_Glc, marking purification as the largest hotspot within production. The strong influence of purification solvents has also been confirmed by Feijoo et al. (2017). From LCA data of different enzyme applications, the influence of fermentation, however, is often described as superior to that of chromatography-based purification. In these cases, washing agents are either not included within the calculations, or purification is performed on a low yielding fermentations process (Becker et al., 2021; Trinidad et al., 2023). Due to the substantial upkeep expense of chromatography columns reported here, filtration may be the preferred method when operating high-throughput enzyme cultivations. Consequently, additional enzyme purification does not yield an environmental advantage and should thus be averted when the intended application of the end-product does not require a high-purity enzyme.

3.3. Environmental assessment of enzyme production on different nitrogen sources

Besides the organic nitrogen sources' substantial contribution to environmental indicators such as ME, LU, OD, GW and photochemical smog formation (Bello et al., 2021; Kim et al., 2009) during enzyme manufacturing, industrial production still relies on organic nitrogen sources, such as soy tryptone and yeast extract. Kim et al. (2009)

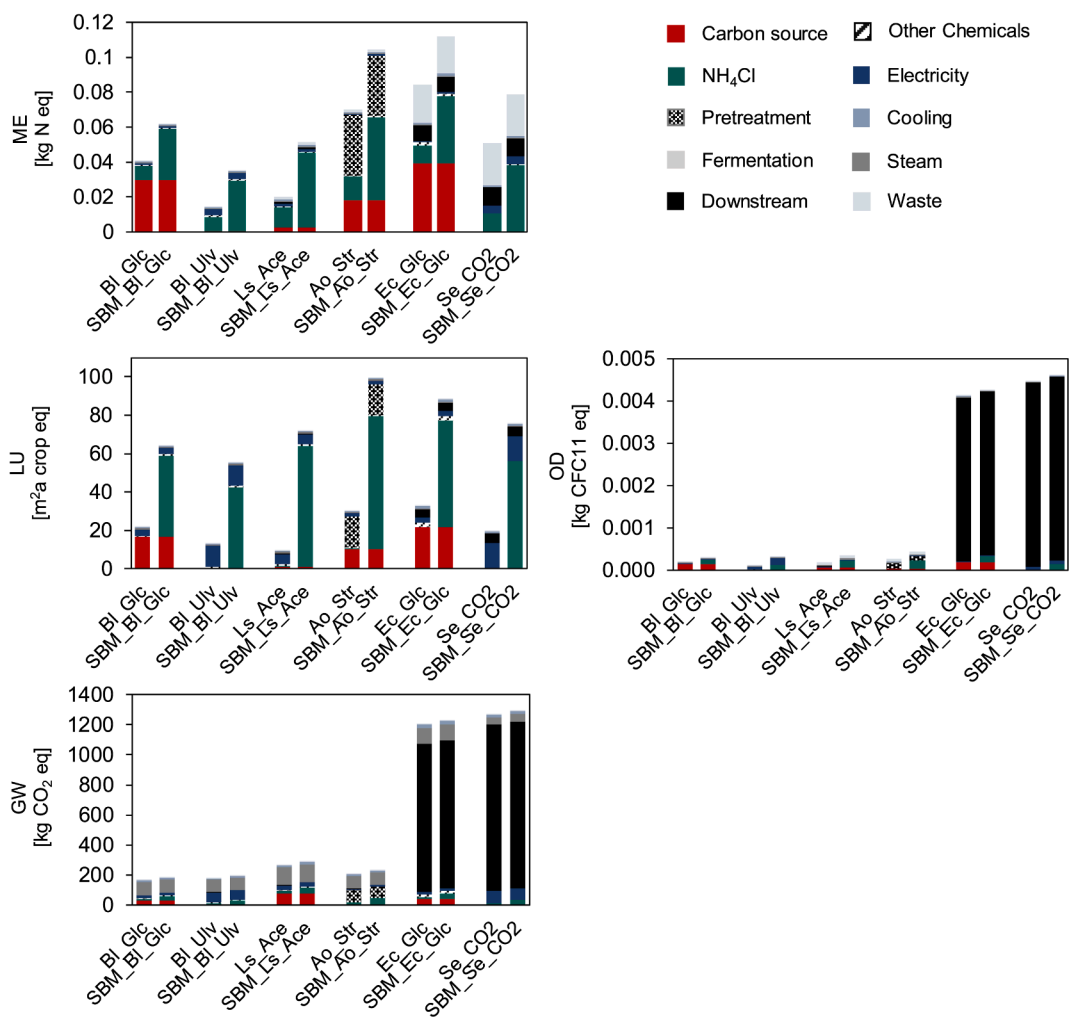

Fig. 4. Effects of replacing the inorganic nitrogen source, here ammonium chloride (baseline scenario) with the organic nitrogen source (SBM) used in fermentation on the ME, LU, OD, and GW impact categories.

reported that during enzyme production 64 % and 72 % of photochemical smog formation stemmed from soybean protein and yeast extract, respectively. In order to assess the potential environmental improvements associated with switching to an inorganic nitrogen source, the environmental performances of soy tryptone (replaced with soybean meal (SBM) from the ecoinvent database) was compared directly with ammonium chloride utilization presented in the baseline rEnz production simulations. While enzyme yields and enzyme activities can be limited by inorganic nitrogen sources, it was assumed that the strains employed are optimized for usage on these nitrogen sources as exemplified in Li et al. (2023), thereby effectively addressing this technical challenge. The comparison revealed that SBM usage increases ME, LU and OD by 3.7-, 331- and 43-fold, respectively, while no significant reduction in GW potential was observed (Fig. 4). These results are validated by the study from Bello et al. (2021), which indicates similar expression for SBM towards these categories. To date, only a limited number of recombinant enzyme LCA studies have taken into account nitrogen sources beyond tryptone or SBM. In an example regarding industrial β-galactosidase production, an inorganic nitrogen source, ammonium sulfate, was assigned 2–3 times lower ME and OD than urea (Feijoo et al., 2017), thus supporting the hypothesis of low-impact inorganic nitrogen. Additionally, the utilization of ammonium chloride in cyanobacteria fermentation exhibited a comparatively lower environmental performance than that observed with sodium nitrate (Johnson et al., 2017), demonstrating further potential of ammonium chloride.

Next to the arable land employed for soybean cultivation or the application of fertilizer and pesticides, transportation further adds to the environmental burden. With Europe itself having nearly no soybean agriculture, the enzyme production industry is highly dependent on imports from abroad, in particular from countries like Brazil (ITC, 2023). Conversely, the ammonium chloride production industry in Denmark is centralized in Europe, specifically in Germany (ITC, 2023), which helps reduce transportation distances and associated environmental impacts. Ammonium chloride is either formed as a by-product of the sodium carbonate process, or directly from ammonia. The latter has reportedly expressed no significant contribution towards LU, ME or OD (D'Angelo et al., 2021), while the impact of the former production route is attributed to sodium carbonate, which overall, allows its favorable environmental performance.

3.4. Environmental assessment of location-specific enzyme production

To assess the effects of manufacturing location on the environmental impacts of industrial enzyme production processes, location specific electricity input and substrate were evaluated. While the North American region dominates the current industrial enzyme market with more than a 30 % share, Asia-Pacific is currently the fastest growing region (Mordor Intelligence, 2022). With leading global companies like Novozymes A/S and Chr. Hansen A/S, Denmark is another key player in the market. Thus, China, the US, and Denmark were selected as locations to be assessed in electricity-focused sensitivity analysis (Fig. 5). Due to data limitations on the majority of the feedstocks utilized, region-specific differences in substrate supply were assessed only for production in and outside of Europe (summarized under US and CN).

3.4.1. Electricity

Electricity is required during different manufacturing steps, *e.g.,* maintaining fermentation conditions, pumping, and supporting cooling operations. The source of electricity, however, varies largely across regions and can consequently exert substantial differences in the overall environmental impact of the manufacturing system. For example, higher HCT emissions are found for China and the US, increasing by 23.1 and 13.5 % for Bl_Ulv, respectively (Fig. 5). Location-specific energy differences are also responsible for an increase in the overall footprint of FPMF, FRS, HNCT, HCT IR, TA, OF, and GW for China and the US (Fig. 5). The main source of energy within these countries is derived from coal in China (65.1 %) and gas in the US (38 %) (Ritchie et al., 2022). The observed results are supported by different studies associating high acidification, HCT, FPMF, and GW with gas and coal, emissions mainly produced during mining operations (Laurent et al., 2017). In contrast, a substantial portion of Denmark's energy mix consists of renewables, such as wind (45 %), and bioenergy (17 %) (Pelkmans et al., 2021). Denmark's bioenergy primarily relies on wood as a resource, which necessitates large amounts of water and land (Mussatto, 2021; Schyns and Vanham, 2019). Fig. 5 illustrates that these factors are prominently more affected by electricity from DK than China or the US. This is evident within an increase of electricity-related WC by a factor of 2, owing to 22 to 148 times higher water requirement for bioenergy compared to oil or coal (Gerbens-Leenes et al., 2008). Additionally, LU is 320.8- and 50.1-fold higher compared to the Chinese or North American system. A comparative analysis from Fthenakis et al. (2009) concluded that biomass energy cycles result in the highest land use per GWh in comparison to wind, coal, nuclear, and solar energy (Fthenakis and Kim, 2009). However, enzyme manufacturing within China had the lowest Co-60 emissions measured in all scenarios, with Denmark being the second highest emitter of radionucleotides − although having no nuclear power infrastructure (Pelkmans et al., 2021). This is due to imported energy from Sweden and Germany, countries with active nuclear energy generation (State 2019) (Mussatto, 2021).

3.4.2. Substrate

The region-specific data entries did not result in significant variations of impacts emitted by Bl_Ulv and Ls_Ace. It is to be noted that no region-specific data was available on glucose. The utilization of straw from Europe increased LU by 70 % (DK − Ao_Str). Within ecoinvent, all straw is summarized under one unit. As reported by the International Grain Council (2023), European straw primarily consists of wheat and maize, while straw from other locations has a higher proportion of soybean and rice. These location-specific variations in straw composition, grains' harvesting, and straw preparation could explain these differences in LU. A second parameter within this process model expresses location-specific distributions. Ammonia-based emissions show different contributions in TA, FPMF, and FE. As an example, TA exhibited a 34-fold increase for outside of Europe-based production (Fig. 5), which can be related to ammonia-based emission released after sugar solubilization during the wheat straw pretreatment phase. As for Europe (summarized under DK), the European Industrial Emissions Directive strongly restricts the emissions of ammonia (European Parliament and European Council, 2010), thus the resulting measurements are consequently reflected within the described environmental flows.

3.5. Limitations of early-stage LCA & future directions

Early-stage life cycle assessments often underlie scalability issues, product's performance uncertainties of specific applications, high data limitations, and insecurity (Zimmermann et al., 2022). To compare deviations among the described scenarios and data uncertainties, Monte-Carlo methodology was applied. The previously reported ineffective translation of direct laboratory energy inputs (Bello et al., 2021), consequently resulting in a considerable increase in impact variability, was partially addressed by employing rigorous bioprocess simulations through SuperPro® Designer. Observed deviations vary across impact categories and are largest within categories where both carbon and nitrogen feedstocks are significant drivers. As stated by Nielsen et al. (2007), impacts can vary between a factor of 10 in some categories such as GW across different enzyme production modes due to differences in productivity, fermentation time and formulation strategies. Employing an optimistic product yield within early-stage production schemes will always be shaped by strong uncertainty. However, it is crucial to note that these specific categories do not serve as a benchmark for evaluating

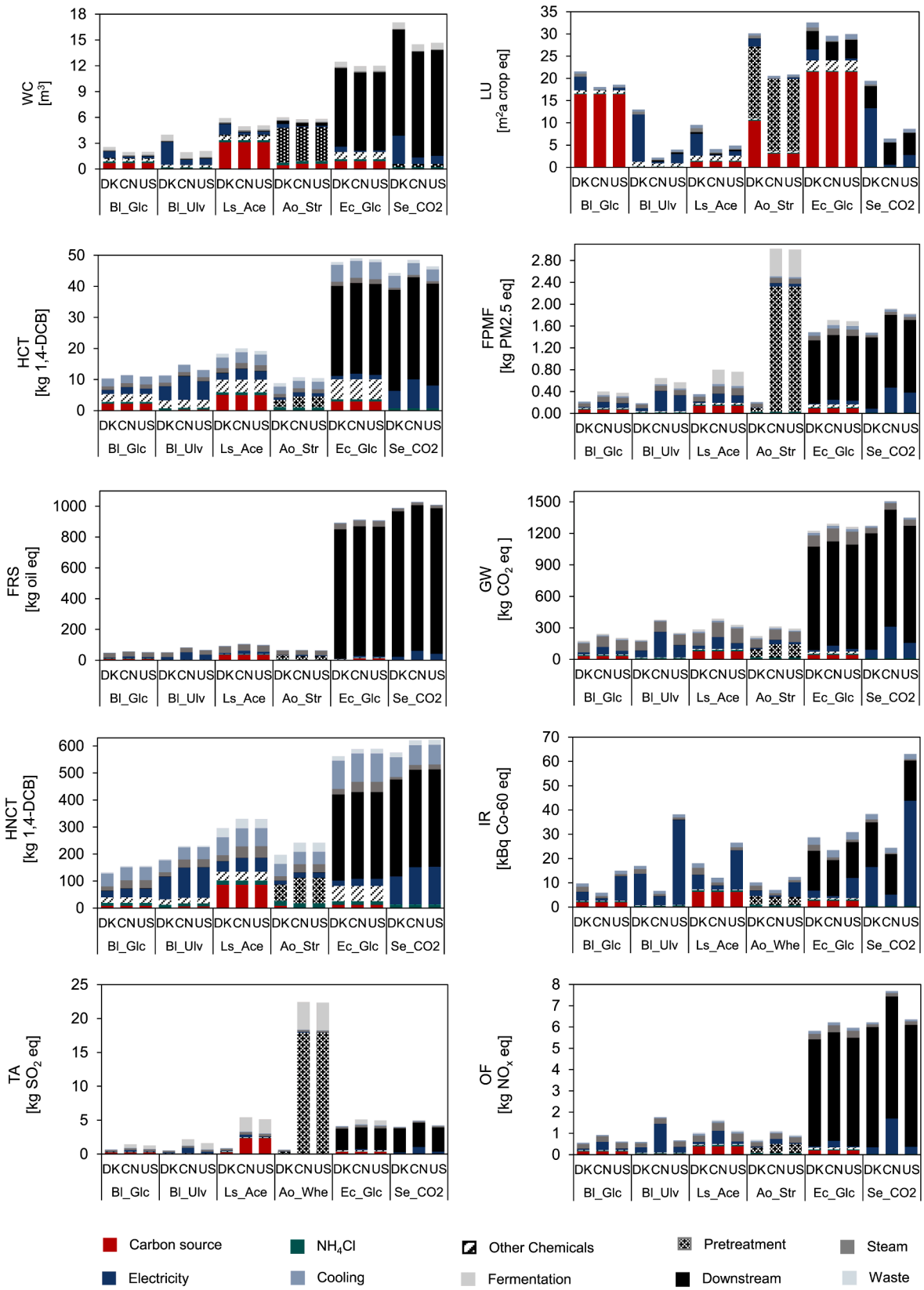

Fig. 5. Life cycle impact assessment for enzyme production in different production locations. Effects are described on WC, LU, HCT, FMPF, FRS, GW, HNCT, IR, TA, and OF (Ozone Formation) impact categories.

the overall sustainability and dispersion within them may not be as pertinent. An additional increase within the intracellular rEnz expression systems can be primarily explained from the chromatography operation, where the lack of available environmental data on washing agents, such as Tris-HCl limits their precision.

It is evident that in order to compete with conventional rEnz manufacturing routes (*i.e.*, Bl_Glc scenario), early-stage low-impact process development should focus on achieving high final-product yields in reasonable fermentation times, as seen in the Bl_Ulv scenario. Although the replacement of glucose with sea lettuce looked promising in reducing the environmental impacts stemming from the carbon source, the slow growth rate of the bacterium on the latter feedstock resulted in longer fermentation times in the simulation, thus driving up the total electricity and cooling agent usage drastically. In similar future scenarios, metabolic engineering or adaptive laboratory evolution to improve the substrate utilization and growth rate of the host organism can be suggested as powerful tools to achieve the desired reduction in environmental impacts. These two approaches, as well as strain discovery efforts, should also prove useful in improving or developing strains that are efficient in secreting target enzymes to the extracellular space, since the purification of intracellular enzymes was shown to result in significantly higher toxic emissions (scenarios Ec_Glc and Se_CO2). Or, as in the Ao_Str scenario, this could allow for partially circumventing the straw pretreatment step. In a similar fashion, discovery or engineering of host organisms that exhibit better growth on inorganic nitrogen sources could allow the replacement of organic nitrogen sources that are widely used in fermentation media, thus allowing a marked decrease in LU or MET (Fig. 4).

4. Conclusion

This study revealed that rEnz production significantly impacts ecosystem and human health toxicity. Unlike prior research, it shows that primary carbon sources not only affect LU, ME, and GW but also contribute up to 25 % of toxic emissions. Alternative feedstocks like *Ulva* or straw could reduce these impacts by 51 % and 64 %, respectively, yet require comparable productivities for environmental competitiveness. Besides enhancing substrate utilization, exploring microbial factories using a single feedstock for carbon, nitrogen, and phosphate to produce enzymes is crucial. A conclusive industrial transformation could prevent pollution, moving us towards greener biocatalysis in alignment with SDG12.

CRediT authorship contribution statement

Mandy Hobusch: Methodology, Investigation, Formal analysis, Conceptualization, Data curation, Visualization, Writing – original draft. **Onur Kırtel:** Conceptualization, Investigation, Project administration, Supervision, Validation, Writing – original draft. **Samir Meramo:** Methodology, Supervision, Writing – review & editing. **Sumesh Sukumara:** Methodology, Supervision, Writing – review & editing. **Ditte Hededam Welner:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.130653.

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