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Advancing biorefinery design through the integration of metabolic models

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ABSTRACT

This study introduces an innovative methodology for early-stage biorefinery design and analysis through the integration of metabolic models into superstructure optimization. The focus is on two types of metabolic models: i) those where ideal growth conditions are assumed, and ii) those where growth is dependent on environmental variables. Metabolic models offer a comprehensive and dynamic representation of the bioreactor system, unbound by the limitations of traditional kinetic models or the need of extensive experimental data. The integration of these models involves a curation and validation process, ensuring that the metabolic capabilities of the microorganisms are accurately represented. Once validated, the models are plugged into the superstructure in the form of surrogate equations that can be handled by the optimization problem for the superstructure. The effectiveness of this methodology is then tested and critically analyzed with the help of two case studies. The primary contribution of this work lies in its effective identification of the most favorable combinations of substrates and microorganisms. The outcome establishes the feasibility of utilizing different substrates and microorganisms in a biorefinery context, thereby highlighting the value of metabolic models in a superstructure optimization framework for exploring early-stage biorefinery schemes. This approach holds significant promise in enhancing the efficiency and sustainability of future biorefinery systems.

1. Introduction

The growing need for sustainable and environmentally friendly production processes has led to an increased interest in biorefineries, which utilize renewable feedstocks to produce a range of chemicals, materials, and fuels (Arias et al., 2023). Designing biorefineries presents a complex challenge due to the diverse range of feedstocks, the variety of alternative technological processes, and the intricacies of interconnecting these processes effectively. Superstructure optimization has emerged as a valuable tool for the design and analysis of such biorefineries, as it enables the identification of the most optimal process configurations and operating conditions (Grossmann and Guillén-Gosálbez, 2010).

In superstructure optimization, each unit process within a biorefinery is represented by a mathematical model, illustrating detailed mass and energy flows and/or transformations within that unit. By formulating the problem in this manner, superstructure optimization enables a comprehensive exploration of potential process configurations, each represented by a unique combination of mathematical models corresponding to the unit processes. The predominant goal is to identify the configuration that optimizes a particular objective, e.g., minimizing cost. Multiple objectives can also be taken into consideration to meet multifaceted sustainability objectives such as minimizing CO2 emissions and production costs simultaneously (Gargalo et al., 2022; Quaglia et al., 2015). Thus, superstructure optimization emerges as a robust computer-aided design tool, facilitating the development of sustainable and cost-efficient biorefineries essential for resource recovery from waste.

In optimizing biorefinery superstructures, accurately depicting the bioreactor phase is challenging. Typically, yields from experimental data or databases mathematically denote mass conversion in reactors (Table 1). While some studies, like those by Vollmer et al. (2022) and Akbari and Barton (2019), have integrated mathematical models for the bioreactor or cultivation stages. Unfortunately, these specific models often neglect the diversity of substrates and microorganisms. Moreover, collecting experimental data for the superstructure or for developing mathematical models is exhaustive, hindering swift evaluations of various substrates, microorganisms, and their effects on biorefinery

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Table 1
Literature review of biorefinery superstructure optimization and the bioreactor stage representation.

Description	Bioreactor model	Reference
An open-source software tool, O2V, for superstructure optimization of biorefinery designs in Denmark.	Yields from experimental data	Gargalo et al. (2022)
Biorefinery designs to produce Sorbitol and xylitol from switchgrass.	Yields from experimental data	Galán et al. (2021)
Identify an optimal processing route to produce bio-succinate among numerous process alternatives.	Yields from experimental data	Garg et al. (2019)
Superstructure optimization of bioethanol production from various raw materials.	Yields obtained from a database	Bertran et al. (2017)
Different biorefinery configurations to evaluate the economic and energy efficiency including stillage valorization to produce bioproducts.	Yields obtained from literature	Ng et al. (2019)
The process design of a biotechnological production process for xylitol.	Yields derived from kinetic models	Vollmer et al. (2022)
Technoeconomic assessment of an algal biorefinery producing various possible bioproducts	Yield obtained from a genome scale metabolic model	Akbari and Barton (2019)
biorefinery platform for valorizing household waste to bioenergy, microbial protein, and biochemicals	Yields obtained from literature	Elyasi et al. (2021)
Evaluation of an integrated microalgae-based biorefinery process and energy-recovery	Yields for microalgae found from literature	Rhee et al. (2021)

efficiency, thereby revealing a research gap in exploiting potential bioreactors. (i.e., how to effectively and efficiently evaluate multiple substrate and microorganisms in biorefinery designs).

To bridge this gap, metabolic models emerge as promising tools, offering a more comprehensive depiction of bioreactor processes within the ambit of superstructure optimization. A metabolic model, in this context, refers to a computational representation of the biochemical reactions and pathways transpiring within a biological organism or system. They are engineered to simulate and analyze the intricate interplay of various metabolites and enzymes integral to cellular metabolism (Orth et al., 2010). Compared to more common unstructured models that use kinetic growth rates (e.g., Monod's equation), metabolic models offer several notable advantages for superstructure optimization. Instead of requiring calibration against experimental reactor data, they derive predictions directly from omics data, which is often easier to obtain. Secondly, they are capable of handling multiple substrates, thereby affording a more comprehensive representation of the bioreactor system (Klamt and Mahadevan, 2015).

In this work, we consider two types of metabolic models. The primary focus is on genome-scale metabolic models (GEMs), which reconstruct the metabolic network based on omics data of an organism. (Kim et al., 2017). GEMs allow for product yields to be predicted solely based on the possible substrates that an organism can consume under ideal growth condition. Currently a wealth of GEMs already exists, with 5897 bacteria, 127 archaea, and 215 eukaryote metabolic network reconstructions reported to date, while new reconstructions can be generated using software platforms like KBase (Arkin et al., 2018; Gu et al., 2019).

Consideration is also given to a second category of metabolic models, specifically those dependent on external operational parameters beyond just the substrate, such as pH. These models are typical for community model (i.e., those describing fermentations of multiple microorganisms) since specific product formation can be driven by certain operational parameters (Regueira et al., 2020). The integration of community models into the superstructure is particularly appealing as they enable fermentations with economic and operational benefits, including the avoidance of sterilization and the ability to process complex substrates like nonsterile waste (Kleerebezem et al., 2015). These models will be further referred to as "consortium models" in the manuscript. A prime example of such a model is that presented by Regueira et al. (2020) where they reconstructed the metabolic network for mixed culture fermentations. This model excels in delineating the yields of various volatile fatty acids as functions of parameters like pH and substrate composition. Another great example is the co-culture model made by Hanly and Henson (2011), which simulates the fermentation of glucose or xylose to ethanol. In this model, the yield is influenced by the timing of the transition from aerobic to anaerobic conditions.

Unfortunately, metabolic models are often complex and difficult to integrate directly into the superstructure, necessitating the development of alternative approaches. Surrogate models have emerged as a viable solution to this problem, as they offer a more mathematically straightforward representation of the bioreactor processes while maintaining an adequate level of detail (McBride and Sundmacher, 2019; Vollmer et al., 2022). An excellent example of the use of surrogate models in superstructure optimization is from the works of Stinchfield et al. (2023). In this work, surrogate machine learning models of unit processes -influenced by operational parameters-are used to engineer the most cost-efficient carbon capture facility. In other words, a good surrogate model for the bioreactor stage should factor in the effects of operational parameters, thereby gaining valuable insights into the reactor's operational conditions and the resultant impact on the biorefinery performance.

To be able to use and effectively integrate surrogate models of bioreactors 2 issues need to be addressed. First, it is crucial to assess the quality of GEMs to ensure their reliability for the superstructure optimization problem. This involves addressing inaccuracies and errors that may arise from a lack of manual refinement or other issues in the model construction process. Second, the development of appropriate surrogate models is necessary to bridge the gap between the complex metabolic models and the simplified superstructure representation. In models where growth is influenced by external variables, the surrogate models should also account for these factors. For example, pH, hydraulic retention time or other environmental conditions impacting bioreactor performance.

As a result, the primary objective of this work is to weave state-of-the-art metabolic models into biorefinery superstructure optimization. This integration aims to reflect the vast possibilities of microbial transformations possible in biorefinery designs. Special emphasis will be placed on the development and validation of surrogate models to streamline this optimization process. The ultimate goal is to create a comprehensive toolset (in the form of a workflow) for optimizing the early-stage design of biorefineries, thereby providing a robust framework for future projects. The efficacy of this workflow will be demonstrated through case studies specifically focusing on the production of propionate. This will pave the way for more efficient, cleaner, and sustainable processes, leveraging the full potential of bioreactors in biorefinery designs.

2. Methodology

2.1. Superstructure model

The superstructure model is divided into various process intervals, which represent specific steps in the biorefinery, such as the reactor stage or the downstream processing (e.g., concentration stage). Each process interval includes all possible alternative unit processes that can be employed at that stage of the biorefinery. Within these process intervals, the unit processes are characterized by a set of generic equations describing the different aspects of a certain process (Bertran et al., 2017).

Following this approach, the superstructure optimization problem can be represented as a set of mathematical equations (Eq. 1-6), describing the objective, the different aspects of unit processes (such as mixing or chemical transformation) and logic rules (responsible for the selection of a path in the process network):

$$max\ f(x, y) \tag{1}$$

$$g(x, y) \geq 0 \tag{2}$$

$$h(x, y) = 0 \tag{3}$$

$$x \in X \tag{4}$$

$$x^{LO} \leq x \leq x^{UP} \tag{5}$$

$$y \in \{0; 1\}^n \tag{6}$$

where f is the objective function, typically representing the generated revenue of the refinery. The continuous variables, denoted by vector x, describe the operating variables (e.g., the flow of mass) within their respective lower and upper bounds x^{LO} and x^{UP}, in a continuous feasible region X. Vector y comprises binary variables, which indicate the selection of a specific unit-process. g and h are the vectors of inequality and equality constraints, representing a series of possible operations that a unit process can carry out. These constraints are described by the same generic equations for each process unit, representing the series of possible operations that include; 1) the mixing of various streams, 2) the consumption of utilities like electricity or chemicals, 3) the transformation and separation of mass, and 4) the generation and cost of waste. For a detailed overview of the generic interval equations the reader is referred to the Supplementary Material S.1.

To represent the bioreactor phase within the superstructure, it is essential to formulate an expression for the yield that describes the conversion of feedstock into products. To this end, metabolic models are transformed into surrogate models to represent this transformation in the optimization problem. Using surrogate models can accommodate not only metabolic models, but also other mathematical models with varying degrees of complexity (e.g., nonlinear kinetic models, enzyme reaction models …). Introducing these surrogates into to the superstructure optimization problem allows us to capture essential features of mathematical models and their external parameters influencing the superstructure model. Fig. 1 illustrates a flowchart delineating the methodology for the development of surrogate models designed to predict bioreactor yields. These surrogate models are constructed based on two unique categories of metabolic models, each of which can be directly incorporated into the superstructure optimization problem via two distinct pathways highlighted in the flowchart. The first type of surrogate model originates from GEMs and is tailored for describing and predicting the metabolism of microorganisms, under ideal growth conditions. The second type of surrogate model focuses on metabolic models, where the yield of products may be influenced by various environmental conditions, such as the pH. These second type of models can be GEMs but also other models which rely on simulating the metabolic networks of microorganisms.

2.2. Metabolic models under ideal growth conditions

To incorporate GEMs into the superstructure that assume ideal growth conditions, one can follow pathway 1 in Fig. 1. The first step (step 1.1) in this path is to select the desired GEMs to be used in the superstructure. GEMs can be found in literature and various databases, such as BiGG Models, BioModels, MetaNetX, and MEMOSys, which provide a rich resource of existing metabolic network reconstructions for different organisms. Furthermore, GEMs can be created for new organisms using platforms like KBase, facilitating the construction of metabolic models based on their sequenced genome corresponding to step 1.2 in Fig. 1 (Arkin et al., 2018). BiGG Models is the preferred database because its models are predominantly validated experimentally and are of superior quality (Zachary et al., 2015). For this study, GEMs from literature were selected as they were more apt for the case studies in focus.

2.2.1. Quality assurance

The second step in pathway 1 (step 1.3) is to check the quality of the selected GEMs. Many GEMs often lack the required quality for reliable predictions and necessitate quality assurance and further manual curation. The quality assurance criteria used in this work include: 1) the percentage of metabolic reactions with correctly balanced chemical elements where at least 95% of the reactions must exhibit elemental balance; 2) the percentage of metabolic reactions with a correct charge balance, where 95% of the reactions are charge-balanced; 3) the percentage of dead-end metabolites (i.e., internal metabolites not participating in internal metabolism), should be limited to no more than 5% of the metabolites; and 4) a leak test, which examines if biomass is created without any substrate. To check these criteria, the open-source software tool MEMOTE is employed, offering a unified approach to ensure the formally correct definition of GEMs (Lieven et al., 2020).

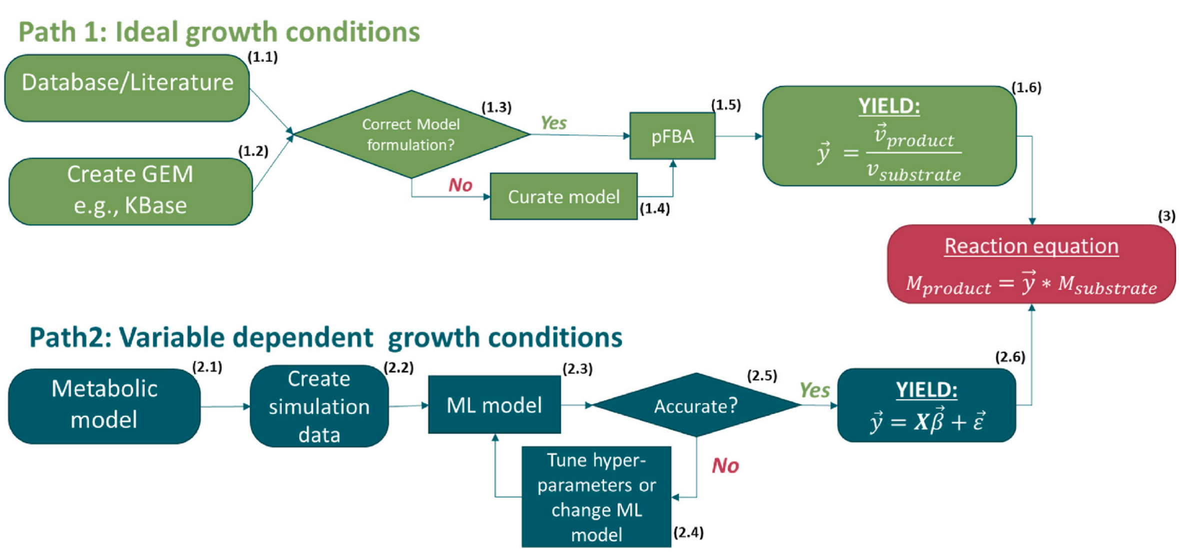

Fig. 1. Schematic representation of integrating genome-scale metabolic models under ideal growth conditions (GEMs) (pathway 1) and metabolic model, under variable growth conditions, into a superstructure optimization (pathway 2), establishing surrogate models for bioreactor yields.

The percentage of dead-end metabolites should be minimized, but they are not necessarily indicators of low-quality models. Nonetheless, a high prevalence of dead-end metabolites (e.g., exceeding 50%) may indicate issues in the reconstruction of the metabolic network, such as improperly connected reactions, necessitating corrective measures. Furthermore, all non-balanced reactions and dead-end metabolites need to be checked and manually corrected if they participate in essential reactions (i.e., reactions crucial for product or biomass formation). To reduce the time dedicated to model curation, only models with at least 95% correctly formulated reactions were considered suitable for integration into the superstructure. Lastly, models producing biomass without substrates indicate a flawed model formulation wherein metabolic reactions are inaccurately interconnected. If any criteria are not met, the model must be manually curated (step 1.4, Fig. 1), as detailed by Thiele and Palsson (2010).

2.2.2. Incorporating GEMs into the superstructure problem

Should the models meet quality benchmarks, the third step in pathway 1 is conducting a Parsimonious Flux Balance Analysis (pFBA) to determine substrate-to-product yields (step 1.5, Fig. 1). pFBA is a computational method used to predict metabolic network behavior. It employs linear programming to optimize the flux distribution of metabolic reactions, aiming to minimize or maximize a desired metabolic flux, typically representing the organism's growth rate. Additionally, pFBA minimizes the total flux through the network, reflecting the concept that organisms have evolved to utilize their metabolism efficiently. By identifying the least biologically "expensive" usage of an organism's metabolism, pFBA offers more accurate predictions of metabolic fluxes, leading to better insights into cellular processes (Lewis et al., 2010; Wortel et al., 2016).

The results of this optimization problem reveal the flux of all secreted metabolites, including the flux at which products are formed, and the flux of substrate consumption. The yield, as a measure of the efficiency of the conversion of substrate to product, is obtained by calculating the ratio of these two fluxes (Eq. (7)):

$$y_{p,s} = \frac{v_p}{v_s} \tag{7}$$

where $y_{p,s}$ is the yield in $g_{product}/g_{substrate}$, and v_p and v_s are the fluxes, in $mg/g_{cell\ dry\ weight}$/hour, obtained from the pFBA of product p and substrate s. This calculation corresponds to step 1.6 in Fig. 1. The yield is used to represent the chemical conversion of a substrate to a product and is placed as a constraint in the superstructure optimization problem according to Eq. (8) (Fig. 1, step 3):

$$M_{r,p} = y_{p,s} \cdot M_{r,s} \tag{8}$$

where M_p and M_s are the flow rates (kg/h) of the resulting product p from substrate s in bioreactor r.

2.3. Metabolic models under variable growth conditions

Fig. 1's flowchart depicts a second pathway for integrating metabolic models into the superstructure, specifically considering growth conditions dependent on variables. Contrary to pathway 1's ideal growth scenarios, this approach requires models to be validated against experimental data, ensuring that model dynamics accurately mirror actual reactor behavior (Fig. 1, step 2.1). Once validated, the initial step in creating a surrogate model involves creating simulation data (Fig. 1, step 2.2), i.e., the yields for various products across different environmental or operational scenarios generated using the original model. The environmental or operational conditions are subjected to uniform and random sampling to achieve a comprehensive representation of the system's behavior across a range of conditions. In the following step (Fig. 1, step 2.3), this data is used to create the surrogate models which are linear or polynomial equations using Ridge regression. Ridge regression is favored due to their ability to prevent overfitting and their straightforward algebraic structure, which aligns well with superstructure optimization algorithms (Hoerl and Kennard, 1970). The surrogate model for consortia models, expressing the yield of a certain product from a specific substrate, is represented by Eq. (9):

$$y_{p,s} = \beta_0 + \sum_j \beta_j \cdot x_j \tag{9}$$

Where $y_{p,s}$ (in $g_{product}/g_{substrate}$) represents the yield of product p from substrate s, β_0 is the intercept, β_j are the coefficients for the predictors x_j representing operating variable j of the reactor. In the presented superstructure model Eq. (9) represents the only non-linear equations in the model. The following step in the pathway (Fig. 1, step 2.4) entails assessing the quality of the surrogate model through the evaluation of parity plots and the Normalized Mean Standard Error (NMSE). If the parity plots reveal discrepancies between the observed and predicted values, or if the NMSE yields high values, adjustments to the surrogate model are most likely necessary. These could involve revising the hyperparameters associated with regularization or contemplating the adoption of an alternative machine learning model, such as Artificial Neural Networks (Fig. 1, step 2.5). If sufficiently accurate, the expression for the yield is used in Eq. (8) (Fig. 1, step 3).

2.4. Building case studies

To showcase the developed workflow, two case studies were built. The superstructures considered focus on the production of propionate using microbial conversions. All superstructures are divided into five process intervals: an input interval, the bioreactor interval, a concentration stage, a selective separation stage and the output interval. In the superstructures, logic constraints are defined to ensure the selection of only one unit process or substrate per process interval. In the subsequent sections, the components and assumptions made for each process interval are elaborated. All final parameters used in each process interval can be found in the Supplementary Materials S3.

2.4.1. Input interval

The GEMs are used to identify possible substrates for the reactors they represent. This identification is automated and is achieved by implementing the following steps: 1) A list of metabolites participating in exchange reactions (i.e., reactions describing the transport of a metabolite from the extracellular compartment to the intracellular compartment) was compiled; 2) All metabolites that are proteins were automatically excluded from this list; 3) Metabolites that failed to produce a minimum flux (>0.10 mmol/gDW/h) of the desired product were also removed; 4) Finally, the remaining substrates were manually examined to determine their suitability.

2.4.2. Reactor interval

The transformation of substrates in the bioreactors is described by the surrogate models of the metabolic models. Additionally, the bioreactor stage assumes that biomass is completely separated from the broth using a filter press (Fricke et al., 2015). In this process interval, waste, being primarily biomass, must be disposed of. As a result, within the bioreactor units, the cost for composting the residual biomass is accounted for (Domini et al., 2022).

2.4.3. Concentration interval

For concentrating the organic acids, two alternative technologies are considered: 1) liquid-liquid extraction followed by a distillation for solvent recovery or 2) The separation of water from organic acids using distillation. The liquid-liquid extraction unit is based on the extraction of the organic acids using hexyl acetate as the organic solvent, which is recovered through a sequence of distillation columns, achieving a high separation yields from water. The separation efficiencies and energy consumption of this process was calculated from the works of Woo and Kim (2019).

The distillation unit is considered as a binary mixture of water and organic acids, where the separation efficiencies and energy consumption are estimated using the shortcut method (Seader et al., 2016). For the calculations and assumptions made for the shortcut method, the reader is referred to the Supplementary Material S.3. The shortcut method is assumed complex enough for the degree of detail needed at this stage, as the primary focus of this study lies on the reactor stage. It should be noted that for further development and refinement of such a biorefinery scheme, a more detailed investigation into effective distillation trains is necessary.

Waste generated from these unit processes predominantly consists of water carrying diluted organic acids, necessitating treatment in a wastewater treatment plant. The economic burden associated with wastewater treatment, as defined by Rodriguez-Garcia et al. (2011), has been incorporated into the respective unit processes.

2.4.4. Selective separation interval

Finally, propionate and acetate are selectively separated into their final products using a distillation unit. Again, the mixture is assumed as binary and the separation efficiencies and energy consumption are estimated using the shortcut method (Seader et al., 2016). The assumptions made for the shortcut method of this process unit, can also be seen in the Supplementary Material S.3.

2.4.5. Objective function

For this study, the objective function of the superstructure optimization problem is to maximize the profitability of the biorefinery design. To calculate the profitability the operating expenses and the gross revenue need to be defined. Considering that waste management and energy consumption costs have been added to the unit processes, an approximation of the operating expenses (OPEX) can be made as defined by Eq. (10):

$$\text{OPEX} = \text{Cost}_{rawmaterial} + \text{Cost}_{energy\mid utility} + \text{Cost}_{\text{wastetreatment}} \tag{10}$$

Where $Cost_{rawmaterial}$ is the cost of procuring raw materials, $Cost_{energy/utility}$ the cumulative costs tied to energy usage and other utilities (such as addition of chemicals), and $Cost_{wastetreatment}$ the financial commitments related to the handling and treatment of waste streams. On the other hand, the gross revenue (GREV) is defined as the profit made from sales of propionate and acetate (Eq. (11)):

$$\text{GREV} = \pi^{\text{propionate}} \cdot M^{\text{propionate}} + \pi^{\text{acetate}} \cdot M^{\text{acetate}} \tag{11}$$

Where $\pi^{\text{propionate}}$ *and* π^{acetate} are the price of propionate and acetate in €/kg and $M^{propionate}$ and $M^{acetate}$ are the total amount of produced propionate and acetate in kg, depended on the flow of mass through the selected process units.

With the profits and expenses defined, the objective of the superstructure can be formulated as the Earnings Before Interest and Taxes (EBIT). In this context, EBIT is defined as the gross revenue (GREV) subtracted by the operating expenses (OPEX) (Eq. (12)):

$$\text{EBIT} = \text{GREV} - OPEX \tag{12}$$

Further details on the parameters used and the build-up of the generic equations of process units can be found in Supplementary Materials S.1. and S.2. The formulation of the superstructure was created using PYOMO (version 6.4.2), in the Python environment (version 3.10.1). The formulation of this problem results in a multiple integer non-linear program (MINLP) and was solved using BARON which is accessible through GAMS (version 37.1.0) (GAMS Development Corporation, 2021).

3. Results

3.1. Constructing the superstructure

Two case studies were successfully created, aiming to i) demonstrate the workflow of integrating metabolic models into superstructure optimization problems; and ii) to highlight the benefits of using GEMs to connect various substrates to possible bioreactors. Both case studies involve a biorefinery producing propionate and acetate considering 24 h of operational time and being able to handle up to 1 metric ton of substrate per hour. Capital investments and thus sizing of the unit processes is not considered. This implies that all unit processes are assumed to be adequately sized to handle the flow throughout the superstructure.

The superstructures of the case studies are schematically represented in Fig. 2. The first case study is deliberately simplified, to offer easily interpretable results. This simplified superstructure serves as a foundation for discussing the methodology of integrating metabolic models. The second case study, by contrast, introduces a greater number of input variables to illustrate the versatility and benefits of using Genome-Scale Models (GEMs) as a tool for evaluating various substrates. This approach not only allows for deeper analysis but also demonstrates how these models can be effectively employed to select the most suitable substrate.

Subsequent sections present the implementation of metabolic models introduced as surrogate models within the superstructure optimization framework, i.e., five GEMs of *Propionibacterium* representing pure cultures, along with a community model representing the mixed culture fermentation model.

3.2. Considered bioreactors

Six bioreactor alternatives are considered in the case studies for transforming the substrate into organic acids (i.e., propionate and acetate). Five of these reactor alternatives are represented by GEMs created by McCubbin et al. (2020), simulating different *Propionibacterium* species: *P. acidipropionici, P. acnes*, *P. avidum*, *P. freudenreichii*, and *P. propionicum*, where ideal growth conditions are assumed. These 5 *Propionibacterium* models were all successfully integrated using pathway 1, i.e., GEMs where ideal growth conditions are assumed. Additionally, one consortium model, representing an open mixed culture fermentation dependent on the pH of the fermentation, is incorporated using pathway 2. This consortium model was created by Regueira et al. (2020) and treats all microorganisms collectively as a single functional entity. This model was not built with a collection of GEMs to describe the consortium, but rather upon the principle of the “enzyme soup” where it is considered that in an open fermentation, all mayor metabolic pathways are available to degrade substrates. The environment however strongly influences the bioenergetics of the fermentation (catabolism, transport, homeostasis …) by selecting the pathway which allows the most energy to be harvested. In this model, the main environmental pressure selecting the metabolic pathways is the pH (Regueira et al., 2020). This consortium model has already previously been validated against experimental data justifying its use in the proposed superstructures. Though the model suggests that various organic acids, such as butyrate and valerate, are produced, it is assumed for simplicity that only acetate and propionate are generated in the mixed culture fermentation, which exclusively consumes glucose. This assumption simplifies the downstream processing and allows us to focus specifically on the integration of the metabolic models in the superstructure. Additionally, the maximum concentration of propionate achievable for pure cultures (represented by the GEMs) is set to 42 g/L. This value is derived from the average titers reported in the literature for fed-batch reactors using *P. acidipropionici* (Ahmadi et al., 2017). To simplify the case studies, it is assumed that all pure cultures under consideration are anticipated to reach a similar concentration of propionate. As the considered bacteria all belong to the same genus, there is a high likelihood of achieving comparable titers. Therefore, this is not expected to be

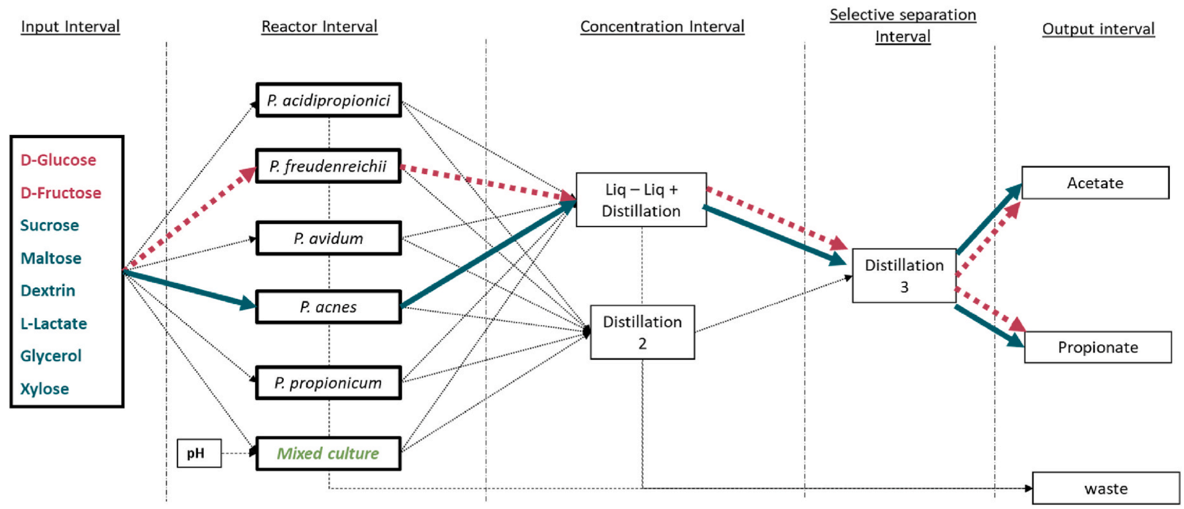

Fig. 2. Superstructure topology representing the two case studies. The red substrates indicate the feedstocks considered in case study 1. The blue substrates are the additional feedstocks considered for case study 2. → indicates the solution of case study 1, → indicates the solution of case study 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the decisive factor influencing the superstructure optimization results. For mixed open culture fermentations, a maximum concentration of 24 g propionate/L is considered, as observed in the fed-batch study by Chen et al. (2016). Based on this information, the extent to which the substrate needs to be diluted can be determined.

3.2.1. *Finding potential substrates*

Following the procedure in section 2.5.1., eight potential substrates were identified for case study 2: maltose, sucrose, lactate, fructose, xylose, dextrin, glycerol, and glucose. However, it should be noted that these substrates represent a pool of options, not all of which can be utilized by every reactor alternative. The actual substrate compatibility varies among the five different reactors represented by the GEMs. For case study 1 only fructose and glucose were selected.

3.2.2. *Pathway 1: curating and incorporating GEMs*

Utilizing the software tool MEMOTE, it was determined that all selected GEMs exhibit high quality, with at least 95% of all reactions being mass and charge balanced. No correction of the unbalanced reactions was needed. Additionally, the percentage of dead-end metabolites was low for all models (<5%) and no biomass was generated from any of the models without substrate, further confirming the quality of the models. However, some corrections to the models needed to be made. First an extracellular compartment was assigned to all models to effectively identify exchange reactions, needed to identify potential substrates. Furthermore, the lower bound for the flux of the cell maintenance reaction needed to be established since it had not been previously defined. In the absence of data to validate the non-growth associated ATP flux, we established a minimum flux of 9 mmol/h/gDW, aligning with the GEM for *Bacillus subtilis* as developed by Oh et al. (2007). This approach is deemed appropriate given that both the considered *Propionibacteria* and *Bacillus* are gram-negative bacteria with the capability to grow in anaerobic conditions.

With the quality of the GEMs assured, the yields for acetate, propionate and biomass were found by taking the ratio of the substrate and product fluxes. The fluxes are obtained by solving the pFBA to maximize the biomass growth rate for each GEM and for each available substrate, under anaerobic conditions. The nutrients used in the simulations of the GEM for each species, can be found in the Supplementary Material S.3. The yields obtained for propionate (being the high value product) using different substrates and microorganisms is presented in Table 2. The yields for acetate and biomass can be found in the Supplementary Material S.3.

3.2.3. *Pathway 2: incorporating the consortium model*

A surrogate model of the open mixed culture fermentation model was successfully integrated into the superstructure. To this end, 60 datapoints were generated by running the community model under varying pH conditions, uniformly and randomly sampled between 4 and 8.5. This number of data points sufficiently spans the input space without needing excessive runs of the community model. Notably, the hydraulic retention time was excluded from consideration due to its negligible impact on the fermentation outcome. A more detailed overview of the simulation settings, of the mixed culture model, can be found in the supplementary materials S3. A 5th order polynomial equation was fitted to these datapoints using Ridge regression, resulting in yield expressions for propionate, acetate, and biomass, with pH as the singular variable. The equations of these regressions can be found in the supplementary materials S.3.

The Normalized Mean Square Error (NMSE) values for the fitted

Table 2
The yield of propionate ($g_{propionate}/g_{substrate}$) for the different substrates and species of Propionibacterium. n.a. indicates that the species cannot consume the corresponding substrate.

species/substrate	*P. acnes*	*P. acidipropionici*	*P. propionicum*	*P. avidum*	*P. freudenreichii*
Sucrose	n.a.	0.31	0.26	n.a.	n.a.
D-Glucose	0.38	0.33	0.28	0.33	0.34
Maltose	0.37	0.31	0.26	0.31	n.a.
Dextrin	n.a.	0.3	n.a.	n.a.	n.a.
L-Lactate	0.55	n.a.	0.43	n.a.	n.a.
Glycerol	0.66	0.56	0.52	0.49	n.a.
Xylose	n.a.	0.36	n.a.	n.a.	n.a.
D-Fructose	0.38	0.33	0.27	0.33	0.40

equations were minimal (6.65 $\cdot 10^{-5}$ for propionate, 1.59 $\cdot 10^{-4}$ for acetate, and 1.77 $\cdot 10^{-5}$ for biomass). Moreover, the parity plots, which compare predicted and observed yields (i.e., generated by the original mixed culture fermentation model), closely aligned (Fig. 3). These findings support the adequacy of the chosen regression technique in replicating the community model, thus endorsing its use within the superstructure optimization framework.

3.3. Results of the case studies

The operation period for the case studies is set for 24 h (assuming operation at steady state), with a capacity to process up to 1000 kg of substrate per hour. The first case study was solved in 2.1 s with an optimality gap of 0.0028. In this case study, only fructose and glucose are considered as potential substrates for the reactor. The optimization results indicate a preference for fructose as the substrate, with *P. freudenreichii* chosen as the organism. The costliest process interval lies in the concentration phase being the liquid-liquid extraction unit. Here the extraction unit accounts for 51% of the operating costs, primarily due to the expenses associated with solvent recovery by distillation. This refinery design proves to be slightly profitable, yielding a daily EBIT of 617 euros (Fig. 4).

In the second case study, an optimal solution was found in 2.8 s with an optimality gap of 0.0023. In this new optimization problem, all possible substrates were identified as detailed in the methodology. The solution now suggests the use of lactate as the substrate and *P. acnes* as the organism for the reactor (Fig. 5). Liquid-liquid extraction remains the chosen unit to concentrate the products and once again constitutes the majority of the OPEX (41%). This refinery design has the potential to generate revenues more than ten times larger than those observed in case study 1 (€8306/day). This is primarily attributed to the efficient conversion of lactate to propionate by *P. acnes.* Propionate, being a more valuable chemical than acetate, enhances the revenue generation capability of this design due to the organism's superior conversion efficiency. A more detailed breakdown of the costs and generated revenues can be found in the Supplementary Material S.4.

4. Discussion

4.1. Optimization results

The selection of substrate and the bioreactor unit exerts a significant impact on the final outcome of the superstructure optimization. Whereas the selection of the unit in concentration stage is negligible. This is because the distillation unit consistently consumes more energy than liquid-liquid extraction, consequently elevating the Operational Expenditure (OPEX) for this stage. As a result, liquid-liquid extraction is invariably the preferred choice for this interval.

Focusing on the substrate and reactor interval, the two critical parameters that drive the optimization problem are the cost of the substrate, which directly affects the overall OPEX, and the yields from the biochemical conversions within the reactors, which impacts the gross revenue. In the first case study, which considers only two substrates (Fructose and Glucose), Fructose—despite being marginally more expensive—is favored due to the higher yields achieved in the reactor containing *P. freudenreichii*. This compensates for the slightly elevated raw material costs, ultimately yielding higher revenues compared to a Glucose-based system. The second case study broadens the scope by evaluating eight different substrates, and the optimal design features lactate as the substrate and *P. acnes* in the reactor. In this scenario, lactate not only ranks as one of the most cost-effective substrates but also produces the highest propionate yields, thus culminating in a financially viable biorefinery design.

According to literature, *P. shermanii* and *P. acidipropionici* are predominantly utilized for propionic acid production. Yields reported in various reactor setups vary, typically ranging from 0.40 to 0.54 g propionate/g glucose (Ahmadi et al., 2017; Payot et al., 1999). This contrasts with yields calculated from the Genome-Scale Models (GEMs), which range from 0.28 g/g to 0.38 g/g. A source of discrepancy lies how the pFBA is formulated, using the biomass flux as the maximization

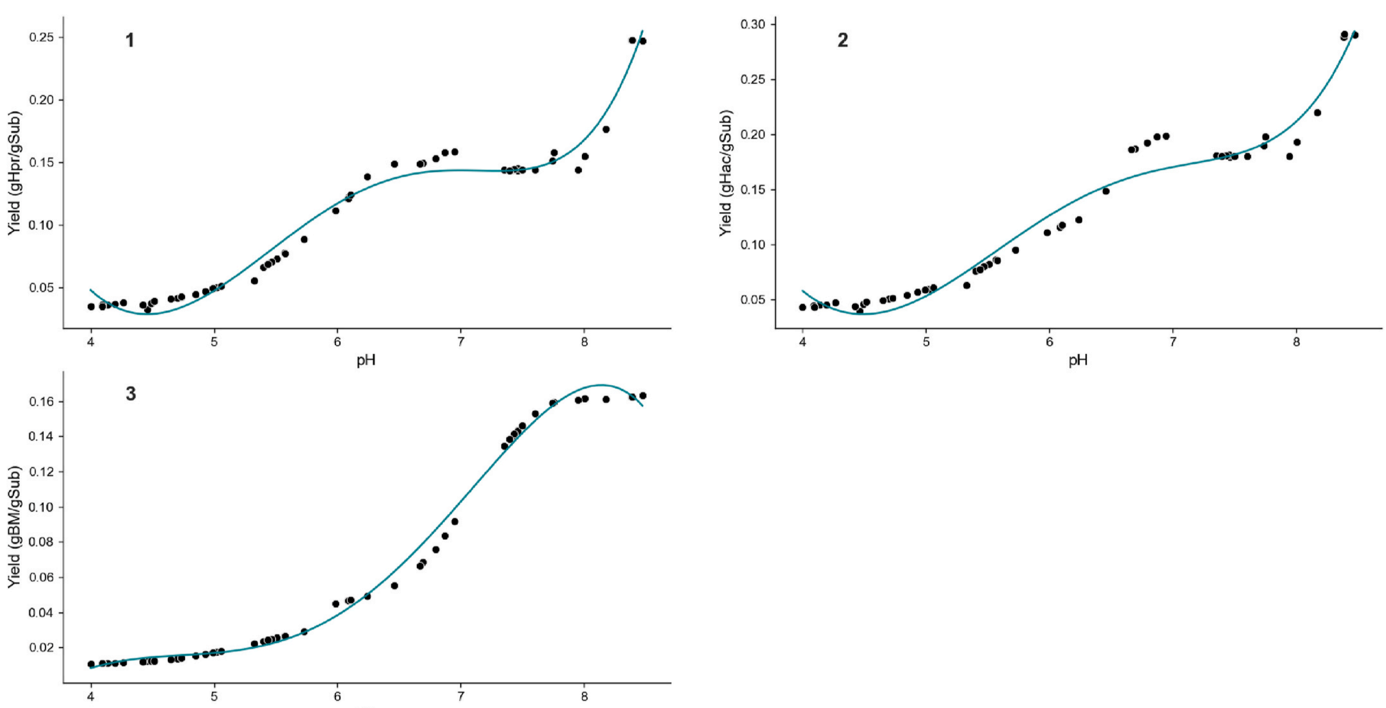

Fig. 3. Surrogate regression models for the yields of the mixed culture fermentation dependent on the operating pH. 1) Yield of propionate (Hpr), 2) yield of acetate (Hac), 3) yield of biomass (BM) as a function of the pH. Solid line represents the surrogate model, dots represent the generated data from the original model of Regueira et al. (2020).

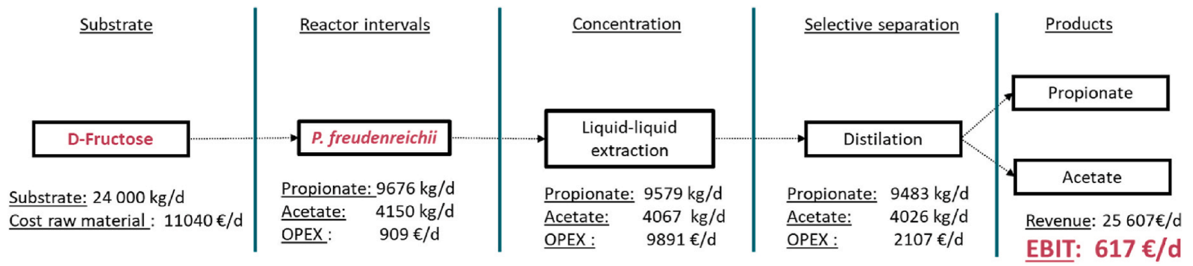

Fig. 4. Optimal process units derived from Case Study 1. The illustration also shows the mass flow of acetate and propionate exiting each unit, as well as the profitability of the design, quantified as Earnings Before Income Taxes (EBIT).

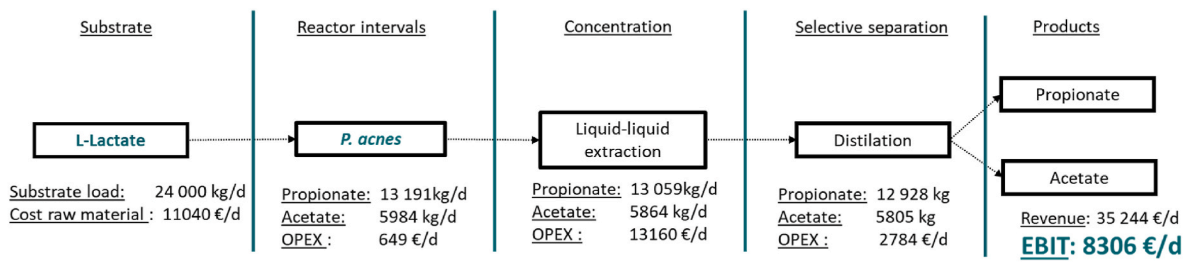

Fig. 5. Optimal process units derived from Case Study 2. The illustration also shows the mass flow of acetate and propionate exiting each unit, as well as the profitability of the design, quantified as Earnings Before Income Taxes (EBIT).

criterion. As a result, consistently lower product yields are expected as carbon fluxes are diverted to biomass instead of to the product. Nonetheless, this criterion is likely to be the closest to a realistic fermentation when no other experimental data are available. Additionally, discrepancies may stem from potential omissions of metabolic pathways in the models, leading to an underestimation of propionate yields. Nevertheless, as the same criterion and standardized methodology (McCubbin et al., 2015), is used consistently for all considered GEMs models, this uniform approach facilitates a fair comparative analysis.

It is also worth noting that in these case studies, the open mixed culture fermentation unit is consistently bypassed in favor of pure culture fermentation units. The primary reason for this is the comparatively lower yields of propionate and acetate achieved through open mixed culture fermentation. However, it is important to acknowledge that open fermentation systems could offer significant operational advantages, such as eliminating the need for sterilization. While these cost considerations are not yet included due to the preliminary nature of this study, they hold the potential to significantly influence future, more detailed investigations into biorefinery design.

In conclusion, these case studies offer compelling insights into the benefits of incorporating metabolic models in superstructure optimization problems. Specifically, this approach allows for: i) the circumvention of the need for experimental bioreactor data for the representation of bioreactor units, and ii) the automated and optimized exploration of a broad spectrum of potential substrates and a diverse set of microorganisms. This methodology, therefore, provides a more efficient and comprehensive framework for biorefinery superstructure optimization.

4.2. Nonlinearity of surrogate models

Incorporating surrogate models, in the form of Eq. (9), into the superstructure can introduce complexities due to their possible non-linear nature. When a multitude of non-linear variable-dependent models are being evaluated, alternative surrogate modelling techniques may provide a more accurate and time efficient solution. For instance, tools like OMLET can facilitate the conversion of neural networks, which excel at describing non-linear relationships, into a Multiple Integer Linear Programming (MILP) formulation. This formulation can then be plugged into the superstructure optimization framework to represent that unit (Ceccon et al., 2022). This transformation is significant because compared to MINLP, MILP problems are generally easier and quicker to solve. Furthermore, in scenarios with numerous non-linearities, this approach can provide more accurate solutions (Vollmer et al., 2022). However, for the presented case studies, such transformations to MILP are redundant and the solver (BARON) successfully finds the correct result in 13.7 s (windows 10, intel core i5-6300, 8 GB ram).

4.3. Limitations and future work

Given that this study serves as an exploratory inquiry into the potential of integrating metabolic models into biorefinery superstructures, it provides a foundational proof of concept. Despite its innovative approach, the framework encounters several limitations warranting future exploration. First, the high-quality model formulation verified by MEMOTE does not automatically ensure model validity, potentially due to inaccuracies like misrepresentations in metabolic pathways. Ultimately, validation hinges on experimental data, underscoring the preliminary nature of this tool in hypothesizing suitable substrate-microorganism pairs for subsequent laboratory validation. In the case of experimentally validated models, the framework can be used as a tool for optimizing process flowsheets.

An additional contribution to this early-stage design framework involves addressing the uncertainty inherent in predictive models. Employing Flux Variability Analysis (FVA) with GEMs could offer a spectrum of feasible yields, adhering to mass and charge constraints. This data paves the way for stochastic optimization, introducing a robust dimension to superstructure optimization by considering uncertainties not just in reactor operations but across various process parameters (e.g., separation efficiencies or feedstock availability). Embracing this comprehensive uncertainty will help in formulating resilient operational strategies, improving the reliability and robustness of future biorefinery designs. Moreover, addressing uncertainty would enable us to perform a sensitivity analysis, to identify critical parameters that significantly impact the results. Understanding these key factors could guide experimental efforts and data collection towards areas of greatest impact and value. Finally, an uncertainty analysis could help quantify the level of risk associated with different design decisions, which could be particularly useful in the early stages of design when decisions can have significant long-term impacts. It would ultimately aid stakeholders to make informed decisions that balance potential rewards with associated risks.

Another area requiring enhancement is the representation of the reactor types in operation, specifically whether they are continuous, fed-batch, or batch reactors. The current superstructure model does not accurately reflect practical scenarios where reactor differentiation in terms of capital investment, detailed operating costs (e.g., cost of sterilization) or sizing are crucial. This simplification overlooks the intricate cost dynamics intrinsic to each reactor type, like the operational efficiency and cost-effectiveness of continuous reactors. Future enhancements should incorporate discrete cost models for each reactor type, considering the sourcing challenges of requisite experimental kinetic data, this task beyond this study's current scope. However, if available, this data could be integrated into the GEMs, enabling determination of reaction durations for batch or fed-batch processes of pure cultures through dynamic flux balance analysis (dFBA). By understanding the process duration, one can e.g., ascertain the frequency of sterilization required. These representations would consider the unique cost structures associated with each reactor type, thereby leading to a more comprehensive superstructure model. For this reason, the current application of metabolic models in the current superstructure optimization framework is most suitable to explore the best potential microorganism(s) in an early-stage design framework.

5. Conclusion

This study presents a novel workflow for the integration of metabolic models—specifically, Genome-Scale Models (GEMs) and variable dependent metabolic models—into biorefinery superstructure optimization frameworks. GEMs provide valuable insights that facilitate the connection between a diverse array of potential substrates and pure cultures. In contrast, variable dependent models offer a representation of mixed microbial cultures under varying operational conditions, which can substantially influence downstream unit operations.

Employing this integrative framework allows for more efficient early-stage design and assessment of biorefinery systems. It enables the exploration of a broader range of microorganisms, substrates, and operational conditions. The utility of this approach is underscored by simplified case studies, which demonstrate the framework's capability to identify the most economically viable combinations of substrates and microorganisms. Notably, the first case study found a slightly profitable designs, while the second revealed a design with a more than 10-fold increase in profits. This was achieved by fully exploiting the range of available substrates for the given microorganisms in the second case study.

In summary, the successful integration of metabolic models into biorefinery superstructures opens new avenues for innovative design strategies. This approach paves the way for the early-stage development of efficient and sustainable bioprocessing systems, fully leveraging the untapped potential of diverse substrates and microorganisms. With a focus on resource recovery from waste, these advanced tools enhance the credibility and sustainability of biorefinery designs, making them more appealing to potential investors and paving the way for tangible project realizations.

CRediT authorship contribution statement

Lucas Van der Hauwaert: Writing – review & editing, Writing – original draft, Software, Methodology, Data curation. **Alberte Regueira:** Writing – review & editing, Supervision. **Miguel Mauricio-Iglesias:** Writing – review & editing, Supervision, Resources, Conceptualization.

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.jclepro.2024.142793.

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