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RESEARCH ARTICLE

Early-stage sustainability assessment of biotechnological processes: A case study of citric acid production

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Abstract

Sustainability assessment using a life-cycle approach is indispensable to contemporary bioprocess development. This assessment is particularly important for early-stage bioprocess development. As early-stage investigations of bioprocesses involve the evaluation of their ecological and socioeconomic effects, they can be adjusted more effectively and improved towards sustainability, thereby reducing environmental risk and production costs. Early-stage sustainability assessment is an important precautionary practice and, despite limited data, a unique opportunity to determine the primary impacts of bioprocess development. To this end, a simple and robust method was applied based on the standardized life-cycle sustainability assessment methodology and commercially available datasets. In our study, we elaborated on the yeast-based citric acid production process with *Yarrowia lipolytica* assessing 11 different substrates in different process modes. The focus of our analysis comprised both cultivation and down-stream processing. According to our results, the repeated batch raw glycerol based bioprocess alternative showed the best environmental performance. The second- and third-best options were also glycerol-based. The least sustainable processes were those using molasses, chemically produced ethanol, and soy bean oil. The aggregated results of environmental, economic, and social impacts display waste frying oil as the best-ranked alternative. The bioprocess with sunflower oil in the batch mode ranked second. The least favorable alternatives were the chemically produced ethanol-, soy oil-, refined glycerol-, and molasses-based citric acid production processes. The scenario analysis demonstrated that the environmental impact of nutrients and wastewater treatment is negligible, but energy demand of cultivation and down-stream processing dominated the production process. However, without energy demand the omission of neutralizers almost halves the total impact, and neglecting pasteurization also considerably decreases the environmental impact.

Abbreviations: CA, citric acid; DSP, down-stream processing; EC, economic constrain; ESSA, early-stage sustainability assessment; GWP, global warming potential; HTP, Human Toxicity Potential; PFD, process flow diagram; R&D, research and development.

KEYWORDS

bioprocess design, citric acid, early-stage sustainability assessment, life-cycle assessment, *Yarrowia lipolytica*

1 | INTRODUCTION

In light of the current bio-economy, sustainability assessment based on a life-cycle approach is indispensable to contemporary process development. In the past decade, many studies have incorporated both environmental and socioeconomic issues in process development [1–3]. This development was motivated by evidence revealing that neglecting environmental and socioeconomic aspects [4] and ignoring significant phases of the life-cycle, such as raw material extraction, manufacturing, and end-of-life, can lead to a non-sustainable design [4,5].

In the early stage of process development, process ideas are translated into a process design, and their functionality is tested via basic process engineering calculations and laboratory experiments. This phase is referred to as the creative stage and is one of the most important stages, given that it determines the overall process features. Important development decisions are usually based on results from early process design [6]. Hence, this phase also represents a unique opportunity for securing the sustainability of the final process [2,3,7].

Liew et al. [8] differentiated three stages of early process design: research and development (R&D), preliminary engineering, and basic engineering. The R&D phase comprises the chemical properties and main process characteristics of several alternative process routes based on laboratory research and available literature. During the preliminary engineering stage, a process flow diagram (PFD) is drafted for the process alternatives using preliminary process flow data. During the basic engineering stage, piping and equipment are designed to implement the requirements defined in the PFD. As reported in ref. 4, early process design consists of the following: conversion process selection and description, flow sheet preparation, preliminary cost estimates, preliminary sustainability assessment, and identification of sustainability criteria when chemical processes are involved. The subsequent detailed engineering stage finalizes the overall process design [7,8].

It is our understanding that early-stage process engineering spans from concept to preliminary engineering, with the aim being to identify and to study novel production processes and their bottlenecks. Concept engineering is based primarily on customer demand from market analysis. This engineering focuses on the necessary and measurable features of a product or service from the customer viewpoint. At this stage, laboratory experiments usually have not been considered. The block flow diagram is a planning tool used in concept engineering.

PRACTICAL APPLICATION

In this paper, an early-stage sustainability assessment of a wide range of substrates of alternative citric acid production with the yeast *Yarrowia lipolytica* was accomplished. The evaluation focused on carbohydrates, alcohols, and triglycerides from primary renewable, fossil, and waste-based resources. Data for the evaluation were gained from comparable cultivations in lab-scale bioreactors. The evaluation supported the selection of promising substrates considering their environmental, social, and economic impacts.

The sustainability assessment method was based on a standardized and widely accepted life-cycle assessment approach using a minimal data set, i.e. data on the substrate, substrate-related yields, and flows for the core bioprocess. The results were determined by the efficiency of the substrate-related yield of the yeast-based CA production on the one hand, and by the duration of cultivation on the other. The method provided an aggregated result for the three pillars of sustainability and ranked possible alternatives.

The preliminary engineering phase focuses on how the product or service specifications may met. It is the first step towards a draft configuration of the process flow [8,9]. During this phase, the process flow is designed based on laboratory experiments, such as shake flask and bench-top experiments. The PFD is the planning tool of the preliminary planning phase and encompasses input and output materials, as well as energy flows. The data gathered for the PFD can be transferred to a list of materials and used to perform early-stage sustainability assessment (ESSA).

Life-cycle sustainability assessment should be performed in the early stage of process design, where it can serve as a precautionary approach and provides a least-cost opportunity for process optimization. This planning is plausible because changes can be made before infrastructure and process details are determined [2,5,10]. Once the process infrastructure is established, the long-term environmental effects are determined [11].

While the preliminary engineering stage is pivotal, the available data are notably limited. Usually, only the process flow is anticipated, and the substrate related yield is defined

by data from the literature or laboratory tests. Thus, data shortages and gaps are clearly the primary challenges in performing a proper life-cycle sustainability assessment-based ESSA.

Numerous methods have been developed in the fields of chemical engineering [1,2,12], mechanical engineering [13], and architecture [10] to assess sustainability in the early stage of process design. However, only a small number of approaches have been developed specifically for process design in industrial biotechnology [14,15]. Heinzle et al. [15] presented a complete sustainability assessment of economic, environmental, and social aspects. These researchers' method includes economic calculations comprising capital and operating cost estimations from experience-based multipliers and profitability calculations in the early stage of process development (basic R&D). Additionally, up-scaling equations based on empirical calculations are provided. The purpose of the environmental assessment is to highlight hot spots to support sustainable process development [15]. Therefore, these researchers' method does not evaluate actual environmental impacts. The main shortcoming of this assessment methodology is that the input data comprise only direct inputs, upstream processes, i.e. indirect emissions are not considered. The environmental importance of inputs and outputs is determined via the environmental factor, which is calculated using the ABC classification method. In this instance, the importance of one environmental impact is classified according to subjective thresholds.

Social aspects are captured via indicators suggested by a survey such as health and safety, quality of working conditions, impact on employment policy, education and advanced training, knowledge management, innovation potential, consumer acceptance, societal benefit, and societal dialogue. For each of these aspects, two types of social indicators are applied for a total of 64 indices. One group of indicators assesses the technology development, while the other group evaluates aspects of technology application [15]. However, these social aspects were not applied in the case studies presented in this literature.

The early-stage environmental evaluation of bioprocesses under uncertainty approach by Gargalo et al. [14] is based on standardized life-cycle assessment (LCA) methodology. This approach refers to the cradle-to-gate life-cycle and can analyze possible production routes with the same product or feedstock in the conceptual or early stage of process development. The calculation is adjusted for handling inventory and parameter uncertainty and data gaps using mass and energy balances and a Monte Carlo simulation technique to fill those gaps and to define the ranges of possible results [14]. Since LCA datasets seldom provide statistics on validity, an expert review method is used to assess uncertainty and to add certain subjectivity to the calculation. The ultimate goal of the methodology is to rank alternative bioprocess development routes according to their sustainability following the steps of an adapted LCA: goal and scope definition, life-cycle inventory, life-cycle impact assessment, external normalization with the Monte Carlo technique as an additional step as well as ranking and selection of processes [14]. Nonetheless, although this methodology is highly practical, it uses subjective expert judgments, is confined to environmental assessment, and neglects economic and social aspects. Finally, the method's applicability is highly limited, since the applied conversions are easily followed only by those with a strong background in higher mathematics.

Due to the above-mentioned limitations in the sustainability assessment methodology, we embark on developing a sustainability assessment (SA) method tailored to the early stage of bioprocess engineering. In this regard, the developed method has to consider both indirect (up-stream) and direct sustainability impacts. It has to assess all the three aspects of sustainability: environmental, social, and economic. Additionally, it should be able to handle data gaps and be improved when the bioprocess is designed in more details. And last but not least, the methodology has to be capable of supporting decision making in process development by aggregating the results of the separate spheres of sustainability and defining clear ranks of alternatives in the sense of relative sustainability.

In the following study, we elaborate on our method and discuss the results of a comparative case study applying this new ESSA method to assess alternative yeast-based citric acid (CA) production with eleven different substrates in different process modes.

2 | MATERIALS AND METHODS

A literature review confirmed that CA is an important, biologically produced bulk chemical with a broad range of applications. With an annual production of 1.6 million tons, the industrial production of CA is currently exclusively realized in a bioprocess with the filamentous fungus, *Aspergillus niger*, using molasses, starch hydrolysates, and other carbohydrates as substrates [16,17].

Non-conventional yeasts, such as *Yarrowia lipolytica*, are also able to produce CA with high product concentrations and formation rates. This yeast species can utilize a wider range of substrates than *A. niger*. In addition to glucose, other carbon sources, such as ethanol, glycerol, vegetable oils, paraffin, by-products, and wastes (e.g. waste frying oil and raw glycerol), can be utilized by *Y. lipolytica* [18–20]. However, the yeast-based CA production process is in an early development stage, since industrial scale applications are not yet established. Thus, this process offers a great case for ESSA.

The goal of our assessment approach (ESSA) is to support decision making toward sustainability [21] from the very beginning of technology development. However, decisions

at the early stage are burdened with the conflict that room for decisions being able to largely influence further developments is tremendous while information on process parameters is very limited. This conflict makes ESSA in bioprocess engineering particularly challenging and requires compromises in detailed data. Thus, our early-stage sustainability assessment approach balances between data scarcity and seminal decisions and introduces a rather gradual approach that should accompany technology development from the early to the final polishing stages.

The early stage comprises the conceptual and preliminary engineering phases, during which the PFD is drafted and continuously extended and detailed. Our ESSA methodology is characterized by the following premises:

(i) ESSA is based on a minimal data set, i.e. data on the substrate, substrate-related yields, and flows (e.g. nutrients) for the core bioprocess.

(ii) ESSA is based on the hypothesis that assessing the core bioprocess provides the most relevant outcome for the overall sustainability of the final bio-production. The impact of the substrate on overall sustainability is a general feature of industrial bioprocesses for bulk chemicals such as CA [22].

(iii) ESSA does not render later assessments obsolete, but marks the first and essential step to securing overall bioprocess sustainability. Not performing an ESSA means that an efficient opportunity for adaptation towards a sustainable bioprocess is unutilized.

(iv) ESSA offers a holistic approach to sustainability, including economic, social, and ecological factors of bioprocess sustainability.

This ESSA methodology is based on LCA, a well-established and accepted sustainability assessment tool following the ISO 14040:2006 standard [14,23,24]. This approach applies a cradle-to-gate LCA approach to capture the input material and energy flows from the PFD and summary table. The product was determined to be the functional unit, and all necessary inputs and wastes are referenced to it. In the case of co-products, the induced environmental effects were allocated among the products along selected allocation rules (e.g. the weight of the products) or were substituted.

ESSA data were collected by experiments conducted in bench-top reactors on the one hand, and from the literature on the other, where process parameters, such as input materials and yield, were collected. To decrease uncertainty and to fill input data gaps, a mass balance, in the form of an input-output table, was produced that served as a calculation inventory.

For early-stage assessment, we chose Global Warming Potential as the only environmental impact because it is the one most applied in LCA, has the greatest political relevance, and correlates strongly with other environmental impacts, such as eutrophication and acidification potential. The impact was calculated as the Global Warming Potential for 100 years, excluding biogenic carbon, using the CML2001–January 2016 characterization model. This calculation was made using the LCA software, GaBi8© with GaBi Professional, GaBi Construction Materials, GaBi Food and Feed, and Ecoinvent databases.

It is notably difficult to assess the social effects of an entire life-cycle in general and even more so in early stages. An important factor to consider, however, is the emission of toxic materials directly affecting human health, since health and safety are important issues both for consumers and workers in the social LCA methodology suggested by UNEP [25]. For our model, this factor is captured by Human Toxicity Potential (HTP), which was calculated using the same characterization model of CML2001–January 2016.

The economics of the process can be calculated as an economic constraint (EC) via the ratio of substrate value to product value because neither the investment nor the operation and maintenance costs are available at this point. The calculation is based on ref. 16 and was modified with the substrate-specific yield to maintain proportionality and to calculate the amount of substrate necessary to produce the functional unit. The calculation follows the function below:

$$EC = \frac{Substrate\ value}{Product\ value} = \frac{C_S \cdot e_S}{p_P},$$

where C_S is the cost of the substrate given in \$/kg, e_S represents the substrate efficiency calculated in kg substrate/kg product (i.e. the inverse of substrate related yield), and p_P is the cost of the product in \$/kg, which is determined by the unit price of the product.

If the EC is less than 1, the financial scope still accounts for the process costs. If the EC is equal to or greater than 1, the value of the possible substrate cost equals or exceeds the revenue, indicating that the scope is not sufficient to finance the process.

As the final step in the method, the environmental, social, and economic results are aggregated. Since the indicators fall into different categories, their aggregation is only possible when they are converted to the same dimension. In our case, we used a ratio scale approach: the simple internal normalization method where the highest number of categories was used as the normalization factor [2,21].

With the internal normalization method, the relative sustainability gains of each sustainability aspect are accounted for in all process alternatives. With this type of internal normalization, each alternative receives a normalized value for its sustainability, including GWP, HTP, and economic constraints. Then, the normalized value of the three sustainability dimensions is totaled and the alternatives ranked according

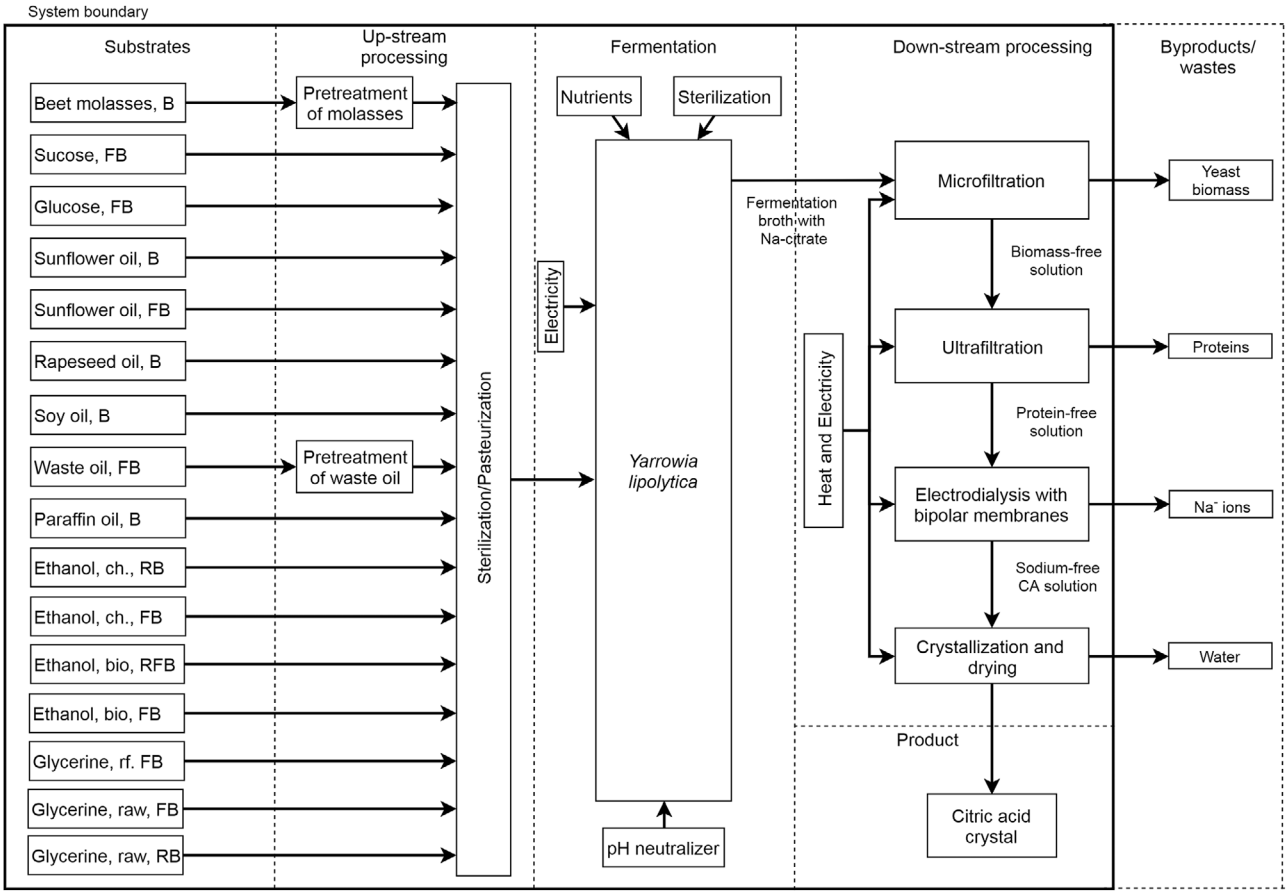

FIGURE 1 CA production alternatives. Key: B – batch process, FB – fed-batch process, RB – repeated batch process

to their overall value. The alternative with the lowest overall value is the most sustainable one relative to the other options.

This ESSA method allows for a simple, holistic, and robust sustainability assessment and fulfills a number of requirements:

(i) The method is based on standardized and widely accepted LCA approach.

(ii) The calculation is based on commercially available and regularly updated databases that comprise both direct and indirect environmental impacts.

(iii) The method provides an aggregated result for the three pillars of sustainability and so supports decision-making. However, it also allows for disaggregation and weak-point analysis, and so it facilitates a more comprehensive understanding of the bioprocess and substrate choice.

(iv) When more data are available for calculation, the model can be developed in tandem with the process and can later be transformed from the early-stage assessment to a complete life-cycle sustainability assessment.

(v) A broad range of different environmental impacts can be assessed with the help of the characterization models in the LCA software.

Concerning the constraints, ESSA results can be used merely to compare the process alternatives with the same scope of assessment determined by the substrates and other core bioprocess constituents. Therefore, our results do not enable a comparison between developed technologies or a fine-tuning of process variables, such as construction materials or alternatives for mixing and aeration.

3 | RESULTS AND DISCUSSION

In our comparative example application of our ESSA method, we gathered and analyzed the early-stage data of sixteen alternative yeast-based CA production processes with *Y. lipolytica*. The data were collected from our own experiments and from the literature of comparable bench top experiments. These yeast-based processes are summarized in Figure 1 that also provides the boundaries of our calculation. In the figure, substrates, nutrients, a neutralizer for pH stabilization and energy inputs both for cultivation and for a simplified down-stream processing (DSP) producing crystallized CA are considered. The DSP was structured as a practicable process line comprising micro- and ultrafiltration, electrodialysis with bipolar membranes, and crystallization/drying for the Na–citrate

containing fermentation broth. The possible substrates, microorganisms, evaluated process modes of CA production and the cultivation times of previous early-stage, benchtop lab tests are summarized in Table 1.

To determine the correct amounts of material and energy flows, a model was built representing a standardized initial and final composition. To this end, the volumetric size was set to 1 m^3, and the model contained a fixed amount of substrate, macronutrients, and micronutrients, with water filling the remainder of the reference volume, that is 1 m^3. The water content for substrates, nutrients, and neutralization compounds was considered using data from the literature [26]. In fed-batch and repeated fed-batch processes, the corresponding amounts of substrate and water added by the substrate were taken into consideration for the end composition. The necessary amount of the neutralizer NaOH was calculated using the temperature-dependent ion fractionation [27].

In the final compound of the fermentation process, the substrate and nutrients are converted to the product, and the necessary amount of neutralizer was added to the solution. As a result, the final volumetric amount was greater than that of the starting compound by the amount of neutralizer added. The product of cultivation was the fermentation broth containing CA and the neutralizer. This was used as an input for the DSP. To adjust these data to the functional unit of our calculations, they were divided by the amount of product CA produced. These data are summarized in Table 2.

The specific energy demand for mixing and aerating the reactors in the cultivation was set to 12 kW/m^3, which is an average of bench top laboratory reactors [28]. The volume of fermentation broth for 1 kg CA (Table 3) was multiplied with this specific energy demand and with the duration of the cultivation process given in Table 1.

In order to model the DSP, data was obtained from the literature. Our DSP model starts with the definition of the volume of the broth after fermentation containing 1 kg CA. The volume was calculated from Table 1 using the given CA concentration. In the next step, the biomass separation by microfiltration was modelled. Since energy demand of microfiltering *Y. lipolytica* on a volume bases was not available, data on specific energy demand of filtering microalgae with 2.125 kWh/m^3 was considered. This value was gained as an average of the available results of two experiments, one with *Chlorella* (1.74 kWh/m^3) and one with *Scenedesmus* (2.51 kWh/m^3) [29]. The volume of the broth calculated in Table 3 was multiplied with this specific value. The volume of the separated biomass was calculated by multiplying the total volume of broth and the biomass concentration in Table 1. This amount was subtracted from the broth considering a maximal separation of 100% and 1.085 kg/L density of biomass [30]. Next, proteins were separated with ultrafiltration where the specific energy demand was taken from river water ultrafiltration, that is, 0.17 kWh/L [31]. This value was gained also as an average of two results, the one with 0.13 kWh/m^3 when the membrane was clean, and the other with 0.21 kWh/m^3 when the membrane was fouled [31]. In the following step, the solution was electrodialysed in a two-chamber system with cation exchange and bipolar membranes to remove Na^- ions. The energy requirement of electrodialysis was set to 0.102 kWh/L gained from own experiments [32]. In the final step, the CA solution is condensed, crystallized, and the crystals are dried. Again, due to data shortages in the literature about this step of the DSP, the energy demand was calculated by using the heat capacity of water, 4.179 kJ/kgK [33], to heat up the liquid from 293.15 to 373.15 K, and the heat of vaporization at 373.15 K and normal pressure, 2257 kJ/kgK [34], to evaporate the amount of water from the solution. The amount of water was taken from Table 2 where it was calculated based on the mass balance of the fermentation process. From this amount of water the weight of biomass was subtracted (see Table 3) and the remaining water was heated up and evaporated. This gives the heat demand of DSP. In this step no heat losses were considered. The total electricity demand was gained by adding the energy demand of microfiltration, ultrafiltration, and electrodialysis.

The environmental assessment was completed with the LCA software, GaBi8©. The production of 1 kg CA was defined as the functional unit. The alternatives were calculated with a parameterized scenario table integrated into the software using the data from Tables 2 and 3. This process enabled high flexibility in scenario development and sensitivity analysis in the model. The quantified results are presented in Tables 4 and 5.

The repeated batch raw glycerol-based bioprocess alternative showed the best environmental performance based on GWP which achieved a high CA concentration of 154 g/L and a relative short cultivation time of 147 h. Although the sunflower oil-base fed-batch process reached much higher CA concentrations (198.5 g/L), due to its long cultivation time (360 h) it ranked only fourth. This fact underlines the importance of time-effective cultivation and the potential of optimizing the high cultivation energy demand of lab-scale reactors. The second-best option was the fed-batch refined glycerol-based process and the third-best alternative turned out to be the fed-batch raw glycerol processes. The least sustainable processes of GWP were those using molasses, chemically produced ethanol, fed-batch sunflower oil, and sucrose.

Human toxicity, the measured social indicator in the early stage, was also calculated with the aforementioned LCA software for the same functional unit. In this study, the waste oil-based CA production was only the third best alternative, and the batch paraffin oil process ranked second. First-best was the sunflower oil-based batch process with moderate CA concentration but with a competitive cultivation time. The soybean oil-based process was the least sustainable process

TABLE 1 Results of early-stage and benchtop lab experiments

	Substrate category	Substrate	Process mode	Microorganism: *Yarrowia lipolytica* strain	pH value	Substrate concentration, g/L	CA concentration, g/L	Substrate related yield, g CA/g substrate	Biomass concentration, g/L	Cultivation time, hours	Source
Renewable	Sugars	Sucrose	Fed-batch	H222-S4(p67ICL1) T5[a]	6.8	150	140.0	0.91	8	191	[35]
		Glucose	Fed-batch	H181[b]	5.0	200	140.0	0.70	7	200	[36]
	Sugar containing by-product	Molasses	Batch	W29ura3-302[a]	5.5	80	50.2	0.61	16	145	[37]
	Alcohols	Ethanol from maize[c]	Repeated Fed-batch	VKM Y-2373[b]	4.5	119.4	105.4	0.883	11.72	144	[18]
		Ethanol from maize[c]	Fed-batch	VKM Y-2373[b]	4.5	138.2	116.8	0.845	11.68	145	[18]
		Glycerol refined	Fed-batch	AWG7[b]	5.5	201	139	0.69	19	120	[38]
	Alcohol containing by-products	Glycerol raw	Fed-batch	Wratislavia 1.31[b]	5.5	200	126	0.63	20	120	[39]
		Glycerol raw	Repeated batch	AWG7	5.5	197	154	0.78	16.5	147	[40]
	Plant oils	Sunflower seed oil	Batch	H181[b]	5.0	70	94.8	1.36	14.1	94	[32]
		Sunflower seed oil	Fed-batch	H181[b]	5.0	170	198.5	1.17	20.5	360	[32,41]
		Rapeseed oil	Batch	H181[b]	5.0	70	98.0	1.40	13.5	98	[32]
		Soybean oil	Batch	H181[b]	5.0	70	95.9	1.37	13	95	[32]
	Plant oil containing waste product	Waste frying oil	Batch	H181[b]	5.0	110	145.0	1.31	16.8	189	[42]
Non-renewable	Crude oil-based	Paraffin	Batch	H181[b]	5.0	89	160.0	1.80	7	200	[36]
	Crude oil-based	Ethanol chemically produced[c]	Repeated fed-batch	VKM Y-2373[b]	4.5	119.4	105.4	0.883	11.72	144	[18]
		Ethanol chemically produced[c]	Fed-batch	VKM Y-2373[b]	4.5	138.2	116.8	0.845	11.68	145	[18]

Yeast genotype.

[a]Genetically modified strain with expression of invertase encoding *ScSUC2* gene of *Saccharomyces cerevisiae*.

[b]Mutant strain.

[c]For calculating two types of industrial ethanol production defined by the authors.

TABLE 2 Material and energy flows of different citric acid production processes with *Y. lipolytica*

	Amounts for 1 kg CA production	Beet molasses [37] Batch	Sucrose [35] Fed-batch	Glucose [36] Fed-batch	Sunflower oil [32] Batch	Sunflower oil [32,41] Fed-batch	Rapeseed oil [41] Batch	Soy oil [41] Batch	Waste oil [42] Fed-batch	Paraffin [36] Batch	Ethanol bio/ch. [18] Repeated fed-batch	Ethanol bio/ch [18] Fed-batch	Glycerine refined [38] Fed-batch	Glycerine raw [39] Fed-batch	Glycerine raw [40] Repeated batch
Input	Substrate, kg	3.9841	1.0714	1.4286	0.7384	0.8564	0.7143	0.7299	0.7639	0.5563	1.1325	1.1834	1.4388	1.5873	1.2821
	Energy of sterilization, MJ	17.8249	6.5321	3.7504	4.1257	1.9704	3.9886	4.0780	2.7145	2.3688			2.8244	3.1434	2.5268
	Energy of Pasteurization [43], MJ				0.0994	0.1153	0.0946	0.0734	0.1029	0.0749					
	Energy of molasses treatment [44], MJ	0.9361													
	H_2SO_4 for molasses treatment [44], g	0.7080													
	$Ca(OH)_2$ for molasses treatment [44], g	0.5348													
	$MgSO_4$, g		1.2214	1.2214	1.8038	0.8615	1.7449	1.7831	1.1875	1.0687	3.2447	2.9280	3.5149	3.8775	3.1725
	$FeSO_4$, g		0.0137	0.0137	0.0202	0.0096	0.0195	0.0199	0.0133	0.0120	0.0013	0.0012	0.0010	0.0011	0.0009
	$(NH_4)_2SO_4$, g		0.0000	0.0000	39.1086	18.6776	37.8316	38.6601	25.7465	23.1719	28.4630	25.6849	0.0000	0.0000	0.0000
	$CaCl_2$, g		0.2143	0.1086	0.1604	0.0766	0.1551	0.1585	0.1056	0.0950					
	KH_2PO_4, g		5.0000	5.0000	7.3840	3.5264	7.1429	7.2993	4.8611	4.3750	9.9620	8.9897	1.4388	1.5873	1.2987
	NaCl, g										4.7438	4.2808			
	$Ca(NO_3)_2$, g										3.7951	3.4247			
	NH_4Cl, g												28.7770	23.8095	25.9740
	Yeast extract, g	19.9203											7.1942	7.9365	6.4935
	Proteose-peptone[a], g	33.8645	2.8571												
	$ZnSO_4$, g		0.0888	0.0888	0.1312	0.0627	0.1269	0.1297	0.0864	0.0777	0.0016	0.0015	0.0012	0.0014	0.0011
	Co, g		0.0037	0.0037	0.0055	0.0026	0.0054	0.0055	0.0036	0.0033					
	Boric acid, g		0.2036	0.2036	0.3006	0.1436	0.2908	0.2972	0.1979	0.1781	0.0054	0.0049	0.0041	0.0045	0.0037
	$CuSO_4$, g		0.0913	0.0913	0.1349	0.0644	0.1305	0.1333	0.0888	0.0799	0.0002	0.0002	0.0002	0.0002	0.0002
	$MnSO_4$, g		0.0895	0.0895	0.1322	0.0631	0.1279	0.1307	0.0870	0.0783	0.0003	0.0002	0.0002	0.0002	0.0002
	MoO_3, g										0.0001	0.0001	0.0001	0.0001	0.0001
	Water in hydrates, g		1.5085	1.5085	2.2277	1.0639	2.1550	2.2022	1.4666	1.3199	3.3966	3.0651	3.6794	4.0590	3.3210
	Water, kg	17.5052	7.1191	7.4150	9.7287	4.6462	9.4055	9.6162	6.4010	5.5857	9.4709	8.5465	6.6601	7.4123	5.9583
Output	NaOH [45], kg	0.4137	0.5728	0.3517	0.3517	0.3517	0.3517	0.3517	0.3517	0.3517	0.2812	0.2812	0.4137	0.4137	0.4137
	Citric acid, kg	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
	Fermentation broth, kg	20.5430	7.2125	7.8733	9.5185	4.5272	9.1695	9.3970	6.1987	5.1724	9.6570	8.7783	7.1436	8.0408	6.2806

[a]Calculations were made with whey protein because protease-peptone was not available in the GaBi database.

TABLE 3 Energy demand of cultivation and down-stream processing

	Volume of initial broth after fermentation	Cultivation	Microfiltration				Ultrafiltration	Electrodialysis	Total electricity demand	Crystallization	
	Volume of broth with 1 kg CA	Energy demand of cultivation	Amount of biomass	Energy demand of biomass separation	Volume of broth without biomass	New CA concentration	Energy demand of ultrafiltration	Energy demand of electrodialysis	Micro-, ultrafiltration, and electrodialysis	Water content of solution	Heat demand of crystallization
	L/kg CA	MJ/kg CA	kg/kg CA	kWh/kg CA	L/kg CA	g/L	kWh/kg CA	kWh/kg CA	MJ/kg CA	kg	MJ/kg CA
Sucrose, FB	7.143	58.937	0.057	0.015	7.090	141.040	0.001	0.725	2.668	6.583	17.058
Glucose, FB	7.143	61.714	0.050	0.015	7.097	140.909	0.001	0.725	2.666	7.472	19.361
Molasses, B	19.920	124.781	0.319	0.042	19.627	50.951	0.003	2.006	7.375	19.811	51.336
Ethanol from maize, RFB	9.488	59.021	0.111	0.020	9.385	106.551	0.002	0.959	3.526	9.265	24.008
Ethanol from maize, FB	8.562	53.630	0.100	0.018	8.469	118.071	0.001	0.866	3.182	8.397	21.760
Glycerol refined, FB	7.194	37.295	0.137	0.015	7.068	141.477	0.001	0.723	2.656	6.593	17.085
Glycerol raw, FB	7.937	41.143	0.159	0.017	7.790	128.366	0.001	0.796	2.928	7.468	19.353
Glycerol raw, RB	6.494	41.143	0.107	0.014	6.395	156.378	0.001	0.654	2.403	5.760	14.925
Sunflower seed oil, B	10.549	42.835	0.149	0.022	10.411	96.048	0.002	1.064	3.912	9.018	23.369
Sunflower seed oil, FB	5.038	78.348	0.103	0.011	4.943	202.323	0.001	0.505	1.857	4.072	10.552
Rapeseed oil, B	10.204	43.200	0.138	0.022	10.077	99.235	0.002	1.030	3.786	8.680	22.493
Soybean oil, B	10.428	42.795	0.136	0.022	10.303	97.063	0.002	1.053	3.871	8.910	23.088
Waste frying oil, B	6.897	56.309	0.116	0.015	6.790	147.280	0.001	0.694	2.551	5.731	14.851
Paraffin, B	6.250	54.000	0.044	0.013	6.210	161.039	0.001	0.635	2.333	4.777	12.379
Ethanol chemically produced, RFB	9.488	59.021	0.111	0.020	9.385	106.551	0.002	0.959	3.526	9.265	24.008
Ethanol chemically produced, FB	8.562	53.630	0.100	0.018	8.469	118.071	0.001	0.866	3.182	8.397	21.760

B, batch; FB, fed-batch; RB, repeated batch; RFB, repeated fed-batch.

TABLE 4 Data and results of economic constraints

Substrates	Yield, kg substrate/ kg CA	Substrate costs, $/kg	Specific cost of substrate, $/kg CA product	Cost of product	EC
Molasses, batch	3.98	0.250[a]	0.996	0.8	1.245
Sucrose, fed-batch	1.07	0.300[b]	0.321	0.8	0.402
Glucose, fed-batch	1.43	0.550[c]	0.786	0.8	0.982
Sunflower oil, batch	0.74	0.794[c]	0.586	0.8	0.733
Sunflower oil, fed-batch	0.86	0.794[b]	0.680	0.8	0.850
Rapeseed oil, batch	0.71	0.829[b]	0.592	0.8	0.740
Soy oil, batch	0.73	0.842[b]	0.615	0.8	0.768
Waste oil, fed-batch	0.76	0.127[d]	0.097	0.4	0.242
Paraffin oil, batch	0.56	1.100[e]	0.612	0.8	0.765
Ethanol ch. prod., repeated fed-batch	1.13	1.100[f]	1.246	0.8	1.557
Ethanol ch. prod., fed-batch	1.18	1.100[f]	1.302	0.8	1.627
Ethanol, bio, repeated fed-batch	1.13	1.100[f]	1.246	0.8	1.557
Ethanol, bio, fed-batch	1.18	1.100[f]	1.302	0.8	1.627
Glycerine refined, fed-batch	1.44	2.000[g]	2.878	0.8	3.597
Glycerine raw, fed-batch	1.59	0.300[h]	0.476	0.8	0.595
Glycerine raw, repeated batch	1.28	0.300[h]	0.385	0.8	0.481
Biodiesel from waste oil	0.70	0.127[d]	0.089	1	0.089

Source: [a] [47]; [b] [48]; [c] [49]; [d] [46]; [e][56]; [f] [51]; [g] [52]; [h] [53].

TABLE 5 Results and aggregation

Substrate	EC	Normalized value of EC	Global warming potential	Normalized value of GWP	Human toxicity potential	Normalized value of human toxicity	Aggregated value	Final ranking
Molasses, batch	1.245	0.346	23.499	1.000	1.626	0.974	2.320	16
Sucrose, fed-batch	0.402	0.112	10.988	0.468	0.668	0.400	0.979	4
Glucose, fed-batch	0.982	0.273	10.200	0.434	0.577	0.346	1.053	6
Sunflower oil, batch	0.733	0.204	8.356	0.356	0.482	0.289	0.848	2
Sunflower oil, fed-batch	0.85	0.236	11.103	0.472	0.620	0.371	1.080	7
Rapeseed oil, batch	0.74	0.206	8.722	0.371	1.229	0.736	1.313	11
Soy oil, batch	0.768	0.214	9.145	0.389	1.670	1.000	1.603	14
Waste oil, fed-batch	0.242	0.067	8.674	0.369	0.530	0.318	0.754	1
Paraffin oil, batch	0.765	0.213	8.630	0.367	0.530	0.317	0.897	3
Ethanol ch. prod., repeated fed-batch	1.557	0.433	11.951	0.509	0.738	0.442	1.383	13
Ethanol ch. prod., fed-batch	1.627	0.452	11.218	0.477	0.698	0.418	1.348	12
Ethanol, bio, repeated fed-batch	1.557	0.433	10.635	0.453	0.611	0.366	1.251	10
Ethanol, bio, fed-batch	1.627	0.452	9.843	0.419	0.566	0.339	1.210	9
Glycerine refined, fed-batch	3.597	1.000	7.682	0.327	0.993	0.594	1.921	15
Glycerine raw, fed-batch	0.595	0.165	8.097	0.345	1.021	0.612	1.122	8
Glycerine raw, repeated batch	0.481	0.134	7.497	0.319	0.890	0.533	0.986	5

followed by that of the molasses and rapeseed oil. Concerning social sustainability, substrates, such as sunflower seed oil, ethanol and glucose, are better options than rapeseed- and soybean oil-based processes, in which the human toxicity impact is approximately two–four times that of the other substrates. The main source of the impact was the application of substrate via emissions, such as polycyclic aromatic hydrocarbons, benzene, and heavy metals. The application of $CuSO_4$ and NaOH

in addition to process heat and electricity consumption also caused a significant amount of emissions of As, Se, HF, NO_x, and C_6H_6.

The details and results of the economic calculation are given in Table 4. The price of CA from waste oil was set to \$0.4/kg due to the constrained application possibilities of waste-based products. The economic performance of the waste oil-based process was the best among those of the different substrates. However, the current alternative of waste frying oil utilization for biodiesel production is still more desirable than using waste oil for CA production. A product price of \$1.1/kg CA was needed to make up the difference. The second- and third-best economic alternatives were production of CA from sucrose and glycerol, respectively. Currently, the most frequently used substrate, molasses, ranks only 11.

For the final step, the environmental, social, and economic indicators were aggregated with the above described simple internal normalization method where the highest number in the category was used as the normalization factor [2,21]. The alternatives were ranked according to their position in ascending order. The results of the aggregation are provided in Table 5.

Because waste oil is the best option for the economic dimensions with a relative high advantage and third best for human toxicity of sustainability, it gains the best ranking. The bioprocess with sunflower oil in the batch mode ranked second with a minimal difference from the waste oil-based process. The least favorable alternatives were the chemically produced ethanol-, soy oil-, refined glycerol-, and molasses-based CA production processes.

Sensitivity analysis was conducted to identify the importance of different parameters, specifically the role of energy demand, nutrients, neutralizers, wastewater treatments, and pasteurization. At first, the main drivers of GWP and HTP are identified. In the results above, both the environmental impact of the substrates and the efficiency of cultivation play an important role. Substrates produced with an energy and chemically intensive process ranked worse. In addition, fed-batch processes with long cultivation times and processes with less product concentration need higher energy demand both for cultivation and DSP, which again had a negative effect on their ranking. This is depicted in Figure 2 where substrates with extreme CA concentrations and cultivation times are listed. In general, it is obvious that the energy demand of cultivation and DSP dominate the GWP. Substrates, like glycerin and sunflower oil, have very high CA concentrations, however, sunflower oil had the highest cultivation time (see Table 1). Soybean oil had only moderate CA concentration but a competitive cultivation time. In contrast, molasses gained the lowest CA concentration and had relatively long cultivation time. The impacts of substrates dominated rather the HTP where different toxic material flows were captured during production of, for example, soy beans and glycerin. However, in case of

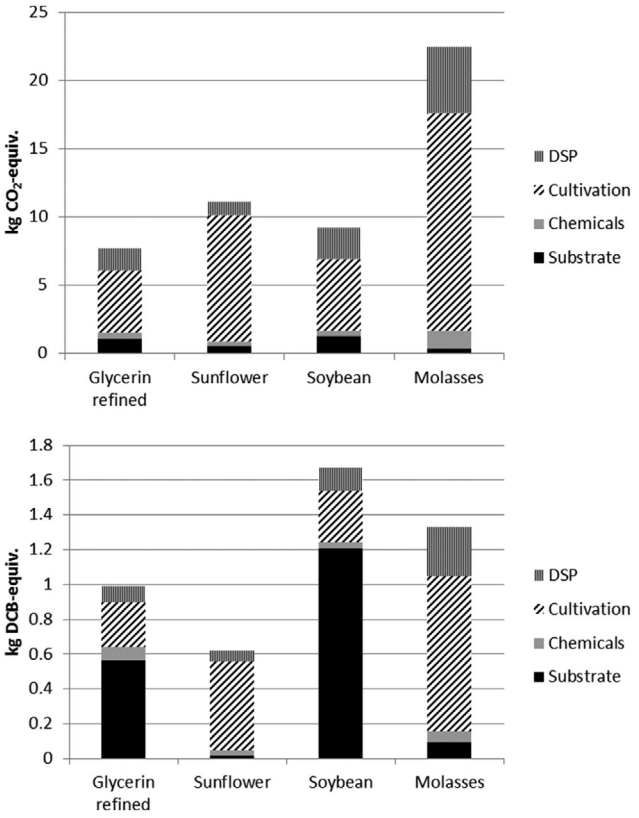

FIGURE 2 Role of different factors in GWP and HTP for selected substrates

substrates with less impact, the energy demand of cultivation and DSP overwhelmed HTP as well, e.g. in case of sunflower oil and molasses.

In the second step, the effects of energy demand were eliminated in order to shed light on the impact of nutrients, neutralizers, water use, and pasteurization were considered. A process without nutrients simulated the possibility of using a medium, such as wastewater, to provide the necessary salts. A scenario without a neutralizer was determined to be more similar to the conventional CA production with *A. niger* and explored the possibility of designing the production process without applying this chemical. In the third scenario, process water was recycled. As a result, wastewater production was avoided. In the fourth scenario, a possible unsterile process was modeled. The environmental impacts of the four scenarios compared to the original calculation of the waste oil-based process for GWP are depicted in Figure 3.

The results shown in Figure 3 demonstrate that the environmental impacts of nutrients and wastewater treatment are negligible. However, neglecting the neutralizer almost halves the total impact, and neglecting pasteurization also considerably decreases the environmental impact without DSP. These indications are quite meaningful for further research on increasing the sustainability of waste frying oil-based CA production.

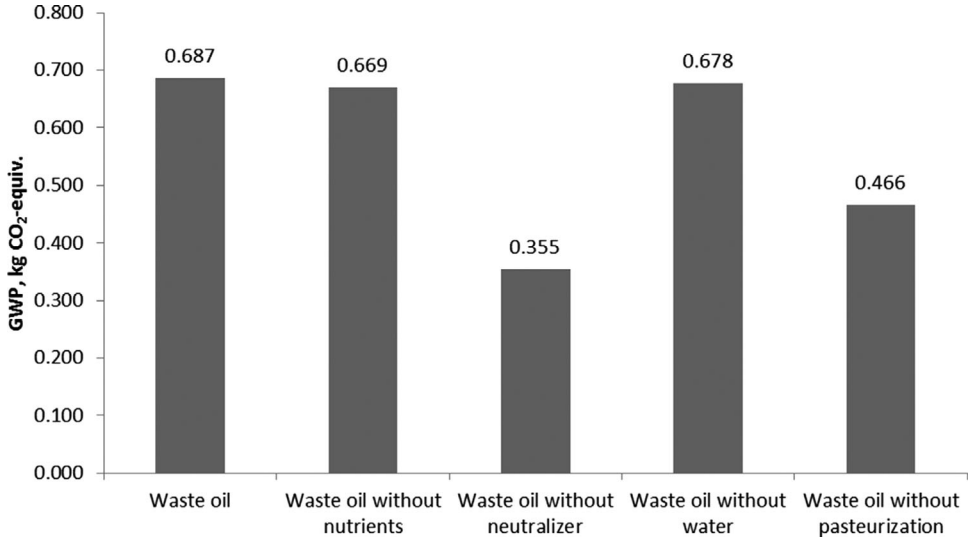

FIGURE 3 Environmental impacts of four alternative processes using waste oil

In general, process optimization with the aim of lowering the required pH value for CA production led to a considerable decrease in NaOH requirements or other pH-adjusting reagents, thereby reducing the GWP. CA extraction without pH adjustment can be achieved through the use of direct capturing methods, such as adsorption or electrodialysis inside the bioprocess, rendering pH-regulating chemicals obsolete. Additionally, sterilization through high energy consumption has a significant GWP. Consequently, an immanent decrease in energy consumption can be achieved through a non-sterile yeast-based bioprocess. However, the biggest potential for increasing sustainability has the reduction of energy demand of cultivation and DSP.

A direct comparison of our results with the literature was not possible, since no other early-stage calculations were made for CA production yet. However, the suitability of produced results can be compared. The only study that compared more bioprocess alternatives and also produced a robust ranking of them was made by Gargalo et al. [14]. We are concerned that this type of presentation of results has an added value to support decision making among the possible alternative bioprocess routes. Nevertheless, the improvement of bioprocesses needs disaggregation of results that is able to highlight hot spots. A well-designed assessment tool should be able to produce disaggregated results and to develop sensitivity analysis that was not demonstrated in ref. 14.

4 | CONCLUDING REMARKS

Owing to the specificities of bioprocess development, early-stage sustainability assessment is strongly recommended. Such ex-ante evaluation enables early identification of future sustainability burdens based on scarce information and assists in process development toward sustainability by ranking available process alternatives according to their environmental and socioeconomic performance.

This paper described a simple but robust early-stage assessment method for bioprocesses based on the standardized life-cycle assessment method, which is widely accepted and for which software and databases are commercially available. With this method, the available material and energy streams were evaluated, the primary weak points could be identified, and possible alternatives were ranked. This method was demonstrated through a comparison of yeast-based CA production alternatives. The results showed that using waste-based substrates provide the ultimate advantage over processes using non-waste substrates. In addition, the minimization of fossil-based energy and chemical additive usage disproportionately improves both the environmental and socioeconomic performance.

In this ESSA exercise, only very limited data on possible process alternatives were available. The data primarily consisted of substrates, the most important additives, and the estimated energy requirements of the main process steps. Material flows for the equipment, specific energy needs, and the environmental impact of the use and end of life phases were not considered during this stage. As a result, ESSA is not applicable for comparing complete technologies, and it can examine only technology alternatives at the same early-stage development level with the same functional unit. However, the assessment model can be incrementally improved with the development of this process as more data become available. Thus, despite the aforementioned limitations, this method provides instructive and useful results for developing sustainable bioprocesses.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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