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On the need of Gate-to-Gate environmental metrics in biocatalysis. Fatty acid

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hydration catalyzed by oleate hydratases as case study.

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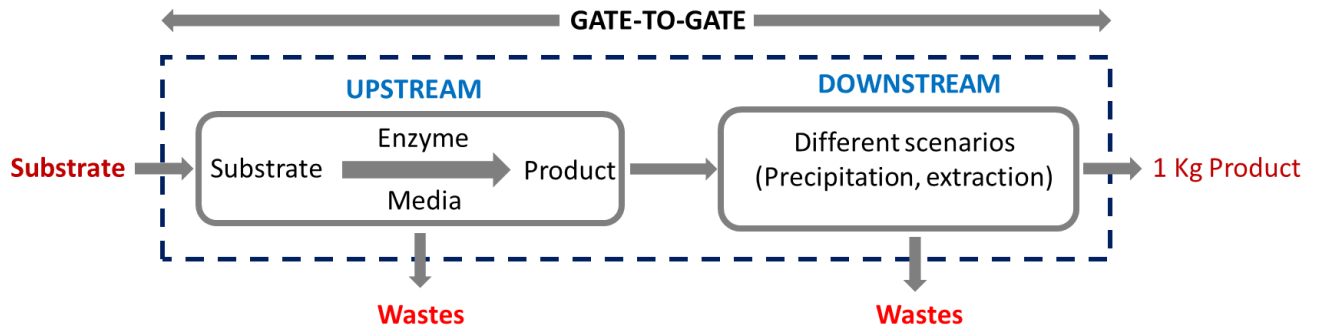
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21 **GRAPHICAL ABSTRACT**



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35 **ABSTRACT**

36 Biocatalysis will play a central role in future chemical processes, aiming at more
37 sustainable strategies while keeping efficiency and economics. The mere use of enzymes
38 does not assure the greenness, and quantitative environmental metrics must substantiate this.
39 Simple but meaningful approaches are needed, particularly when reactions are still at a
40 preliminary phase of research. This paper explores the use of E-Factor and a gate-to-gate
41 strategy to assess environmental metrics for biocatalysis at proof-of-concept stage. The
42 upstream part – the actual biocatalytic reaction – and the downstream – the purification of
43 the product – are considered, providing the complete E-Factor. As case study, the hydration
44 of oleic acid catalyzed by oleate hydratases (OHY) is used. Depending on the reaction
45 conditions (particularly, the substrate loadings), and the chosen downstream, total E-Factors
46 range from 15-20 to 160, mostly comprising wastewater (from the reaction media) and
47 solvent (from the downstream). The estimation of the Total Carbon Dioxide Release (TCR),
48 Kgs of CO₂ per Kg of product, provides a comparable data to benchmark synthetic strategies.
49 The timely identification of hot spots (large contributors to the environmental impact)
50 enables researchers to propose experiments to show that biocatalysis can be performed at
51 more sustainable conditions.

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53 **Key-words:** Green Chemistry metrics; Biocatalysis; Gate to gate; E-Factor; Wastewater;
54 Oleate hydratases.

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57 **1.- Introduction.**

58 The XXIst century is witnessing the establishment of the Green Chemistry principles
59 and the quest of sustainable processes.¹ The need of implementing more benign systems at
60 industrial level, pressured by environmental concerns, is stimulating the research and
61 development in those areas, covering academic and industrial initiatives. Nowadays, new
62 chemical processes must be not only economic or efficient, but also need to fulfil the
63 environmental demands. Derived from that fact, the question that has then emerged is how
64 to determine whether a process is green or not, and which parameters need to be assessed to
65 reach a fair comparison among strategies. Starting from the mere qualitative use of the Green
66 Chemistry principles as basis for the assessment,² the scientific discussion has evolved to the
67 need of defining some quantitative metrics, which can serve as reliable standards for
68 industrialists, academics, and for the society. However, assessing the greenness of a process
69 is often complex and requires the analysis of many parameters, being usually time and
70 resource consuming. The boundaries for the assessment can be placed differently, and thus
71 more or less variables need to be addressed. For instance, a gate-to-gate analysis focuses on
72 a reaction as such, considering the impact of the synthesis and isolation of the product,
73 assuming that reagents, solvents, catalysts, etc., are already placed in the chemical plant, and
74 their environmental impact is not covered. Conversely, a broader cradle-to-gate approach
75 would also assess the origin of the raw materials, the land consumption to produce them, the
76 transportation impact, and the process as such. Furthermore, even more holistic Life-Cycle-
77 Assessments (LCA) take into account the entire life cycle of a product (e.g. cradle-to-grave),
78 and further aspects need to be considered, including the product use and ultimate disposal.
79 Admittedly, the broader the assessment, the better to assure that the environmental impact is

80 adequately considered. However, for daily basis research at laboratory scale the resources to
81 conduct such holistic assessments may not be available, and simpler but still meaningful
82 alternatives need to be developed. In that respect, while many processes are “labelled” as
83 green just by qualitatively following the Green Chemistry principles, some quantitative
84 parameters and strategies have been proposed.²⁻⁶ The Sheldon’s E-Factor parameter is surely
85 one of the most used ones, due to its intuitiveness and simplicity of use.⁷⁻⁸ Basically, the E-
86 Factor represents the kilos of waste produced per kilo of product, thus reporting a rapid hint
87 on the environmental impact of a reaction. While initially excluding water from the
88 assessment, more recent works tend to determine the complete E-Factor, where “everything
89 but the product” is included. Importantly, the E-Factor can be distributed between processing
90 units (up- or down-stream), or among synthetic steps, or be allocated for chemicals and
91 solvents, thus providing a clear picture on what parts or materials are contributing more to
92 the environmental burden. This gives options to concentrate the efforts in improving the hot
93 spots. The E-Factor has recently been complemented by adding the influence of the energy
94 in the environmental impact, and the E⁺-Factor has been proposed.⁹

95 Among the emerging technologies aiming at establishing more sustainable chemical
96 processes, biocatalysis is expected to occupy a central role, as many implemented industrial
97 processes already showcase.¹⁰⁻¹¹ Modern enzyme technologies are versatile, and enable the
98 use of different reaction media – from aqueous to different organic solvents or even solvent-
99 free systems –, and in batch or continuous devices, what significantly enhances the
100 opportunities to integrate biocatalysis in industrial set-ups.¹²⁻¹⁴ However, the mere use of
101 enzymes does not warrant, as such, that a process can be regarded as green or sustainable. In
102 fact, environmental quantitative metrics for the process need to be provided to substantiate

103 this.¹⁵⁻¹⁷ Despite this, however, the critical assessment of the environmental impact of a
104 biocatalytic reaction is not a widely established approach yet, and literature examples are
105 somewhat scarce.¹⁸⁻²³ Furthermore, the environmental assessments tend to focus just on the
106 upstream part – that is, the synthetic step catalyzed by an enzyme –, but often overlook the
107 downstream unit, where the formed product is purified to be ultimately marketed. Actually,
108 the assessment of both parts is equally important to validate how green a process may be, and
109 to determine which actions can be proposed to improve it.

110 This paper proposes a straightforward and rapid assessment of Green Chemistry
111 metrics when biocatalytic reactions are still at laboratory scale, and systems can be optimized
112 keeping environmental standards in mind. The timely determination of the hot spots – that
113 is, what factors are contributing mostly to the environmental impact – may help researchers
114 establish experiments to diminish the waste production and/or to demonstrate that greener
115 conditions are possible. To this end, a gate-to-gate approach is conceived, covering both
116 upstream and downstream units, and depicting different scenarios to understand the
117 importance of the reaction conditions and the downstream choice in the ultimate
118 environmental impact. Complementing the gate-to-gate approach, an analysis of the Total
119 Carbon Dioxide Release (TCR) is conducted as well. The TCR parameter has been recently
120 proposed and addresses the total amount of CO₂ that would be generated in the worst-case
121 scenario, if the product and all the wastes are incinerated.²⁴ It provides a defined and
122 comparable unit to evaluate the actual environmental burden that the process may generate.

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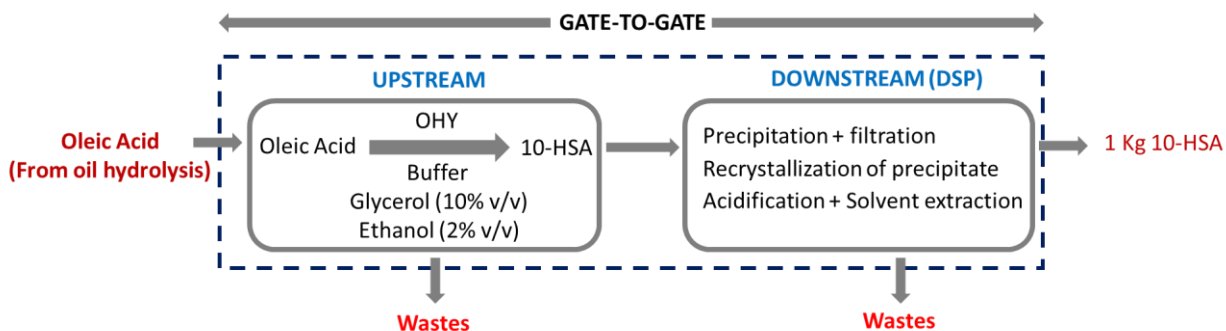
124 **2.- Case study. Oleic acid hydration catalyzed by oleate hydratases to produce**
125 **10-hydroxy-stearic acid (10-HSA).**

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127 The hydration of oleic acid catalyzed by oleate hydratases (OHY), to afford 10-
128 hydroxystearic acid (10-HSA) was selected as model process. The reaction was described
129 almost one century ago,²⁵ and some microorganisms able to perform the hydration were
130 isolated in the 60s.²⁶ In 2009 the actual enzyme was characterized,²⁷ and in recent years
131 processes with industrially-sound conditions for the hydration of fatty acids have been
132 reported (e.g. high substrate loadings and productivities).²⁸⁻³⁰ Several updated comprehensive
133 reviews discuss the topic in details.³¹⁻³³ Despite its potential future industrial use, no
134 environmental studies on this reaction have been published so far.

135 As the first step for the gate-to-gate analysis, the “functional unit” was defined. The
136 emphasis is put in the reaction and product isolation, and it is assumed that all reagents,
137 enzymes and auxiliaries are already in the chemical processing plant (“gate”), and their
138 associated environmental impact in their production and transportation is not considered.^{34,35}
139 The aim is the production of 1 Kg of the desired product (10-HSA), which is delivered in a
140 marketable (purified) form at the exit of the chemical plant (“gate”). Thus, the approach
141 enables the analysis of the environmental impact of the reaction as such and may help
142 researchers in the quest of more environment-friendly reaction conditions during their
143 laboratory work. Importantly, the functional unit is separated in two main sub-parts, as both
144 may be relevant in the environmental assessment, First, the upstream part, where the actual
145 biocatalytic reaction takes place, converting substrates into products, and generating

146 associated wastes. Second, the downstream unit renders the desired product in the pure,
147 marketable form, and further associated wastes are generated as well (Figure 1).



148

149 **Figure 1.** Functional unit for the gate-to-gate assessment of the hydration of oleic
150 acid catalyzed by oleate hydratases (OHY) to generate 1 Kg of 10-HSA. The environmental
151 impact associated to the production of chemicals and catalysts, as well as to the consumed
152 electricity is not considered in this stage.³⁴⁻³⁵

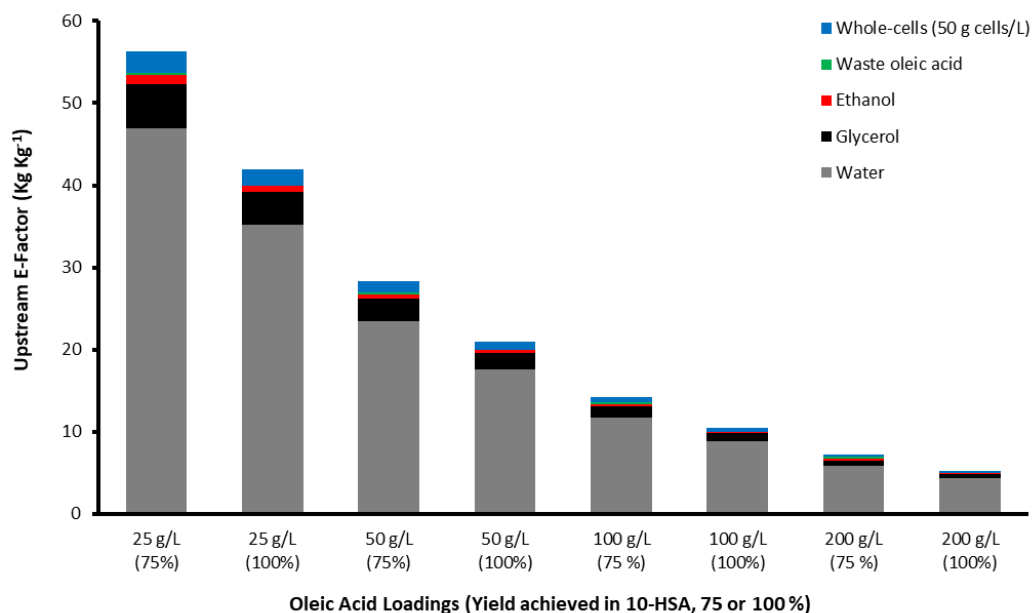
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154 For the oleic acid hydration (upstream), reaction conditions reported in literature,
155 which are believed to be close to industrial applications, were taken into account.³⁶⁻³⁸ To that
156 end, an aqueous solution was employed as reaction media, with a co-solvent mixture
157 composed of glycerol (8 % v/v) and ethanol (2 % v/v). Under those conditions, it is reported
158 that 10-HSA precipitates in the form of a white solid.³⁶⁻³⁸ Herein, for the downstream
159 processing, three scenarios were considered: i) simple filtration of the precipitated 10-HSA,
160 which is only washed with water and dried; ii) as the precipitated 10-HSA contains some
161 traces of oleic acid, a subsequent recrystallization step is suggested, using hexane: ethyl
162 acetate (2:1); iii) in a different approach, acidification of the reaction media with HCl and

163 extraction of 10-HSA using ethyl acetate (3X), and subsequent addition of different
164 chemicals (NaCl, celite, sulphates) to render the pure 10-HSA (Figure 1).

165 By looking at the reaction set-up in qualitative terms, it could be said that the synthesis
166 can be considered to be a green process. Thus, the atom economy is excellent, and a biogenic
167 substrate is used (oleic acid), which comes from oil hydrolysis, and it is readily available
168 without the need of many intermediate cracking steps.³⁹ The process proceeds in water as the
169 paradigm of a non-hazardous reaction media, and both glycerol and ethanol (the cosolvents)
170 and the enzymes are biogenic and biodegradable too. The reaction proceeds at mild
171 temperatures and pressures as biocatalysis is typically displayed. Unlike hexane (see below),
172 the process uses ethyl acetate as solvent for extraction, which is recommended in most of the
173 solvent rankings reported in the literature.⁴⁰⁻⁴⁴ Herein, the (misleading) interpretation would
174 be that no further Green Chemistry analysis should be needed for this synthesis as the
175 principles are fulfilled. However, a more in-depth evaluation involving quantitative data
176 draws a different conclusion (see below). The timely assessment of the environmental impact
177 may help establish experiments and developments that can contribute to greener processes.

178 First of all, the upstream part (the synthetic reaction) was analyzed. To that end, the
179 mixture water-glycerol-ethanol was set as reaction media,³⁶ and loadings of oleic acid in the
180 range of 25-200 g L⁻¹ were assumed, according to most of the prominent examples reported
181 in literature when industrial applications are envisioned.^{28-33, 36-38,45} Whole-cell biocatalysts
182 overexpressing OHY were used (range of 50 g cells L⁻¹). Two yields were assumed, 75 % as
183 a realistic framework, or 100 % as best-case scenario. The contribution of E-Factor of the
184 different chemicals and (co)solvents is depicted in Figure 2.



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186 **Figure 2.** Contribution to the E-Factor of the upstream part, depending on the
 187 substrate loading (25-200 g L⁻¹) and on the final yield in 10-HSA, 75 % or 100 %.

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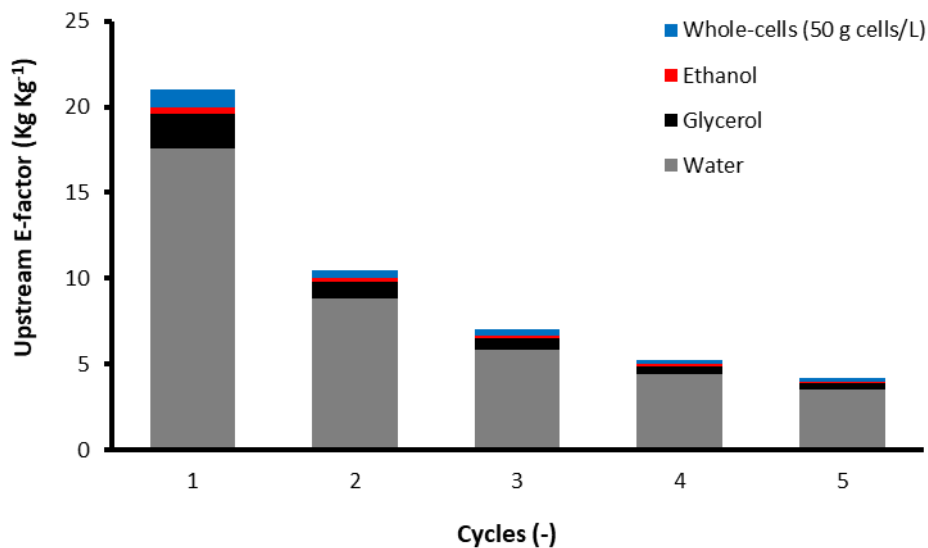
189 As observed, the waste generation is significantly higher at low substrate loadings,
 190 with E-Factors in the range of 60 when 25 g L⁻¹ of oleic acid are added, at 75 % yield. When
 191 the reaction is assumed to proceed at more “industrially-sound” conditions (range of 100 g
 192 L⁻¹),¹⁵⁻¹⁷ the process leads to more decent E-Factors (range of 10). Therefore, intensifying the
 193 reaction (in terms of substrate loadings) may provide beneficial effects both for the
 194 economics of the process as well as for the environmental burden associated to it.¹⁷ Given
 195 the reaction conditions, the hugest contribution to the E-Factor is wastewater (water
 196 containing glycerol, ethanol, traces of fatty acids, cells, etc.), which will need to be treated
 197 before disposing it to the environment. Water shortage is becoming an increasingly important
 198 problem worldwide, and thus the adequate treatment of water effluents needs to be carefully

199 considered. In biocatalysis, the use of other water-free systems may be an alternative to
200 reduce water consumption.^{12,17,46} Finally, it must be noted that the environmental impact
201 derived from different yields (75 vs. 100 %) results more significant at low substrate
202 loadings than at intensified systems, giving further arguments to the idea of enhancing
203 substrate loadings to reach better economics and environmental prognosis.

204 Although it is obvious that intensifying systems are beneficial, it must be said that
205 reaching high substrate loadings is not always accessible in synthetic reactions. Solubility
206 problems, enzyme inhibitions, or by-product formation may appear. To cope with this, an
207 option is to evaluate the possibility of recycling the reaction media (in this case, the
208 wastewater containing glycerol and ethanol) for several cycles. In Figure 3 a simulation is
209 performed, assuming a substrate loading of 50 g L⁻¹, 100 % yield, and several reaction media
210 reuses. As it can be observed, the environmental impact is considerably ameliorated in the
211 first three cycles, leading to upstream E-Factors in the range of 7 (mostly wastewater). Albeit
212 repeated recycling may be challenging, it may be realistic that at least two-three cycles could
213 be reached in many biocatalytic applications (assuming 10 % water loss between each cycle).
214 Surprisingly, not many papers deal with aqueous media recycling,^{17,47} and it is certainly
215 recommended that some experiments are included in academic research to validate an
216 improved E-Factor for enzymatic reactions when recycling is achieved. Likewise, the set-up
217 of continuous processes with media recirculation may lead to highly integrated devices that
218 may combine economics with decent-to-excellent environmental metrics as well.^{13,17}

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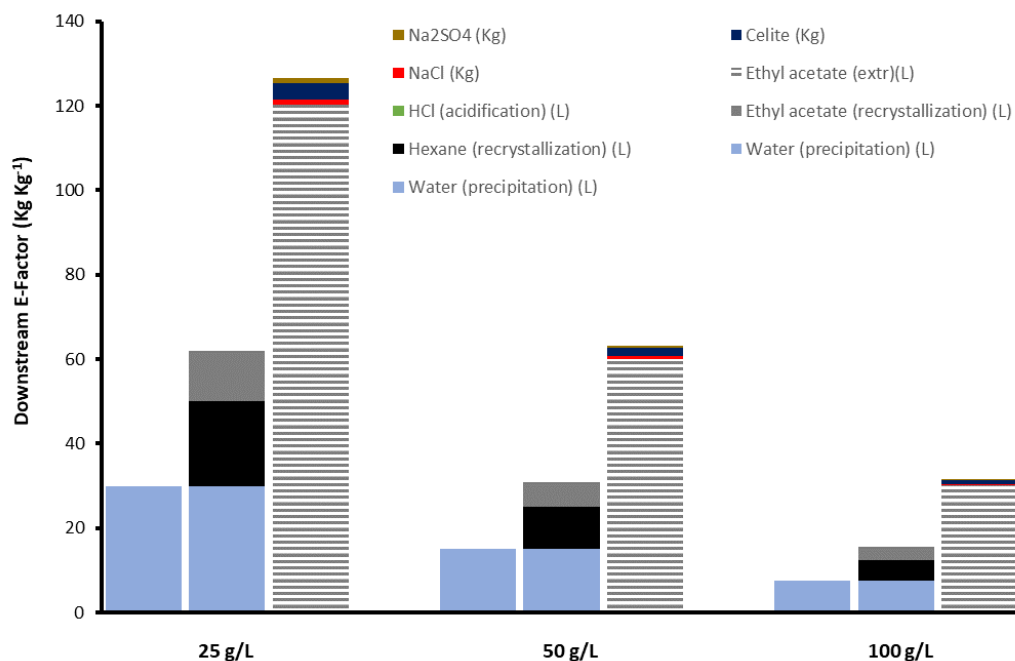


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222 **Figure 3.** Upstream E-Factors observed when reaction media are reused several
 223 times, assuming oleic acid loadings of 50 g L⁻¹, and 100 % yield.

224

225 Once the upstream part was studied, the different options related to the downstream
 226 processing were subsequently assessed. As stated above, three main scenarios were
 227 considered for the downstream.³⁶⁻³⁸ In the simplest one, 10-HSA precipitates from the
 228 reaction media, and a simple filtration and washing with water is assumed (Figure 4, first
 229 column). Connected to that, and to remove traces of oleic acid present in the precipitated
 230 product, a subsequent recrystallization step was included. In this case, the precipitate is
 231 dissolved in hexane: ethyl acetate (2:1) and crystals are obtained upon solvent evaporation
 232 (Figure 4, second column).³⁶ Finally, in a different approach, the aqueous reaction media was
 233 acidified to pH 2 with HCl, and extracted with ethyl acetate (3X), which upon evaporation
 234 and drying rendered the 10-HSA (Figure 4, third column).



235

236

Figure 4. Environmental analysis of downstream options, according to literature,³⁶⁻³⁸

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and at different 10-HSA loadings (25-100 g L⁻¹). First column: precipitation and washing;

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second column: after precipitation and washing, subsequent recrystallization step using

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hexane: ethyl acetate (2:1); third column: acidification and extraction of the reaction media

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with ethyl acetate (3X).

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Results depicted in Figure 4 are explanatory on the significant impact that the

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downstream unit may have when it comes to environmental metrics. While most research

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papers dealing with biocatalysis only refer to the E-Factor related to the synthetic upstream

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– in publications where environmental metrics are provided –, the contribution of the

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downstream to the impact is often not considered. The environmental impact is particularly

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relevant when popular extractive methods are applied, as a surplus of extractive solvent (3X)

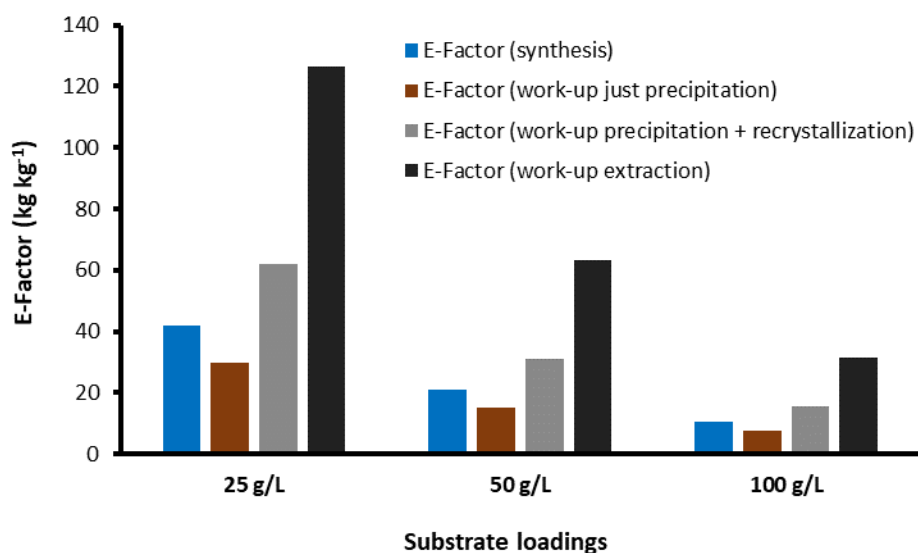
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leads to rather high E-Factors. As an example, at substrate loadings of 25 g L⁻¹, the extractive

249 E-Factor is in the range of 130 (mostly related to solvent use). Solvent recycling (assuming
250 10 % loss per cycle) may certainly be mandatory to reach better figures,¹⁷ and some
251 experiments at laboratory scale would be recommended to validate that option. However, it
252 must be noted that when extraction is intended, the previous acidification of the reaction
253 aqueous media may hamper its further recyclability, as an acidic wastewater effluent is
254 generated. The subsequent wastewater treatment of the acidic effluent may add further
255 environmental impacts. From a different perspective, the situation results different when
256 other downstream units are foreseen (Figure 4). Thus, the precipitation + washing step leads
257 to wastewater formation (presumably with traces of reaction media, oleic acid, 10-HSA, etc.)
258 which may even be considered for recycling (as washing agent for two-three cycles). In that
259 case, E-Factors in the range of 30 can be observed (Figure 4, no recycling). When the process
260 is combined with the recrystallization step, the environmental burden created by the solvents
261 increases the E-Factor to the range of 60. The environmental impact improves significantly,
262 once again, when higher substrate loadings are considered. Thus, at 100 g L⁻¹ more
263 reasonable E-Factors in the range of 10-15 may be reached for those approaches. Finally,
264 while ethyl acetate is a recommended solvent for virtually all ranking lists, and may have a
265 biogenic origin, the use of hexane adds significant (hazardous) environmental burden to the
266 approach.⁴⁰⁻⁴⁴ This timely identification may help find alternatives for recrystallization that
267 could avoid such solvents. Likewise, recovery and recycling of the crystallization solvents
268 appears as a must as well if environmental concerns are considered. Laboratory experiments
269 can show the validity of this option at early states of the development.

270 Once both parts of the functional unit – upstream and downstream – are assessed
271 (Figure 1), the total E-Factor for the production of 1 Kg of 10-HSA was summarized (Figure

272 5). Following the criteria, different substrate loadings were modelled, and upstream and
273 downstream contributions were separated to provide a clear overview of the actual
274 contributions of each part.



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276 **Figure 5.** Total E-Factor for the production of 1 Kg 10-HSA when different substrate
277 loadings are set, and several scenarios for downstream processing are considered.

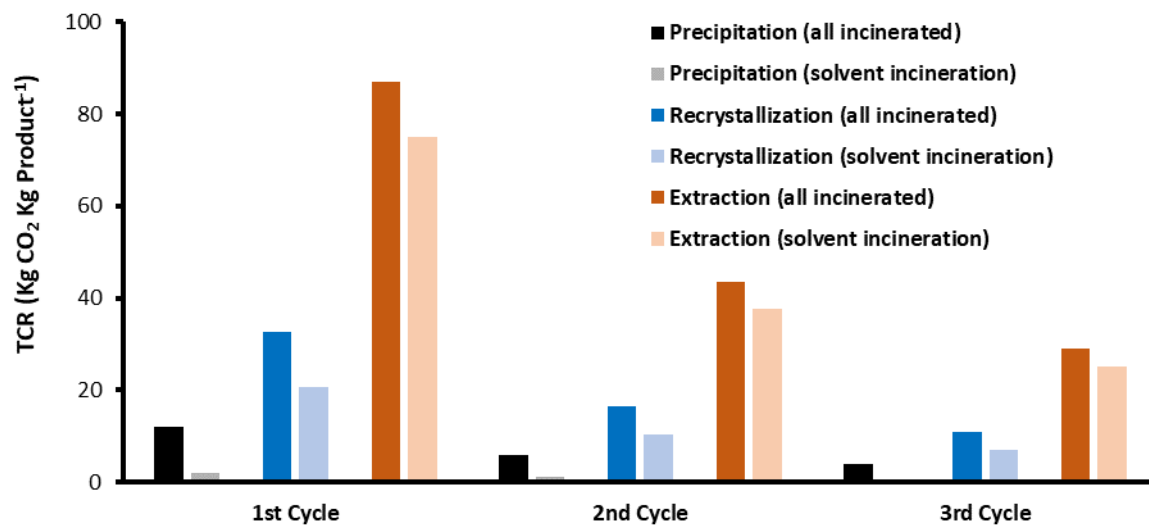
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279 Two main waste streams are produced in the process, wastewater (coming from the
280 reaction media), and solvents (eventually coming from recrystallization or from extraction).
281 Cumulative E-Factors are in the range of 160 if loadings of 25 g L⁻¹ are set, and extractive
282 methods are used without recycling. To put it in clearer numbers, this implies 160 Kg of
283 waste per Kg of the desired product (10-HSA)! The environmental burden results more
284 acceptable at higher substrate loadings, with E-Factors in the range of 15-20, depending on
285 the chosen options. If recycling systems are applied, more acceptable figures can be expected,

286 although, as noted above, the previous acidification step in the extraction alternative may
287 hamper the recycling of the reaction media in that case.

288 Wastes are produced in any (bio)chemical process, and they must be placed
289 somewhere in our planet. Thus, some considerations on the fate of the wastes need to be
290 made. Herein, two main working lines can be depicted, one for the wastewater effluents, and
291 another one for the organics (mainly solvents). The first one is treated in different wastewater
292 treatment units, and finally released to the environment. The second one is ultimately
293 incinerated, generating CO₂ as the main product. In some cases, when wastewater results to
294 be highly recalcitrant, incineration for that fraction is considered as well. A recent paper
295 proposes the use of the “Total Carbon Dioxide Release” (TCR) concept, as Kg CO₂ produced
296 per Kg product.²⁴ In this way, each synthesized product can have a particular “CO₂ currency”,
297 which can enable better comparisons among products, syntheses, etc. The TCR is calculated
298 assuming the worst-case scenario, in which both the wastewater and the solvents are
299 incinerated, and the product (1 Kg) is included as well, as it is assumed that sooner or later it
300 will be converted to CO₂ again. Following this useful theoretical approach, for the oleic acid
301 hydration system, the TCR was calculated considering two options, either incineration of all
302 wastes, or incineration of the organic part only, as it can be assumed that a wastewater
303 containing cells, glycerol, ethanol, and rests of fatty acids should be treated efficiently with
304 milder approaches than incineration. The system is modelled assuming a substrate loading of
305 100 g L⁻¹. To further illustrate the importance of recycling, reaction media and solvents, the
306 systems was simulated involving several recycling cycles (Figure 6).

307



308

309 **Figure 6.** Determination of the Total Carbon Dioxide Release (TCR) considering
 310 either the incineration of all wastes (water and organics), or only the organic part, and
 311 assuming several recycling loops of solvents, reaction media, etc. Data estimated for oleic
 312 acid loadings of 100 g L⁻¹.

313

314 Interestingly, data retrieved from the TCR analysis follow the same patterns as those
 315 obtained for the E-Factor. Assuming the extractive approach (one single use of solvents and
 316 water), the observed TCR is in the range of 90 Kg CO₂ Kg product, while TCRs in the range
 317 of 10 are assumed when the precipitation approach is taken (washing with water only). When
 318 wastewater is not incinerated, the TCR improves in all systems. It must be noted, however,
 319 that probably some fossil fuel energy will be used for the wastewater treatments, and thus
 320 intermediate values between the two values could be expected. Likewise, it must be noted

321 that some of the released CO₂ will be biogenic (e.g. coming from the substrate, use of
322 biogenic (co)solvents, etc), and thus the actual impact of CO₂ release may be more limited in
323 this particular case. The TCR determination results an excellent tool to validate (bio)catalytic
324 processes at laboratory scale, and to be able to put forth better environmental alternatives
325 when research is performed.

326

327 **6.- Concluding remarks.**

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329 Biocatalysis has excellent prognoses to become a green and sustainable alternative
330 for future chemical processes. However, the mere qualitative application of the Green
331 Chemistry principles is misleading, as not all enzymatic processes – or in other words, not
332 all reaction conditions – are green *per se*. Using the E-Factor and performing a gate-to-gate
333 analysis of the intended reaction, carefully covering both upstream and downstream
334 environmental impacts, may help researchers identify hot spots to improve, and to show that
335 actually greener reaction conditions may be reached. In general, intensification of the
336 process, in terms of substrate loadings may align industrial interests with environmental
337 impacts. Moreover, a “culture of recycling” must be created in the community, to show at
338 laboratory scale that reaction media and solvents can be reused for several cycles. Finally,
339 the Total Carbon Dioxide Release (TCR) analysis is an excellent tool to compare different
340 synthetic approaches, and to pinpoint the proper conditions with limited environmental
341 impact.

342

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346

347 **References**

- 348 1. V.G. Zuin, I. Eiks, M. Elschami, and K. Kümmerer, *Green Chem.* **2021**, *23*, 1594-1608.
- 349 2. P. Anastas, and N. Eghbali, *Chem. Soc. Rev.* **2010**, *39*, 301-312.
- 350 3. B.M. Trost, *Science* **1991**, *254*, 1471-1477.
- 351 4. K. van Aken, L. Streckowski, and L. Patiny, *Beilstein J. Org. Chem.* **2006**, *2*.
- 352 5. M. Eissen, and J.O. Metzger, *Chem. Eur. J.* **2002**, *16*, 3580-3585.
- 353 6. C. Jiménez-González, C.S. Ponder, Q.B. Broxterman, and J.B. Manley, *Org. Proc. Res.*
354 *Dev.* **2011**, *15*, 912-917.
- 355 7. R.A. Sheldon, *Green Chem.* **2017**, *19*, 19-43.
- 356 8. R.A. Sheldon, *ACS Sust. Chem. Eng.* **2018**, *6*, 32-48.
- 357 9. F. Tieves, F. Tonin, E. Fernández-Fueyo, J.M. Robbins, B. Bommarius, A.S. Bommarius,
358 M. Alcalde, and F. Hollmann, *Tetrahedron* **2019**, *75*, 1311-1314.
- 359 10. G. de Gonzalo, and P. Domínguez de María (Eds), *Biocatalysis: An industrial*
360 *perspective.* **2018**. RSC Publishing, Cambridge, UK.

- 361 11. G. de Gonzalo, and I. Lavandera (Eds), *Biocatalysis for practitioners*. **2021**. Wiley-VCH.
362 Weinheim. Germany.
- 363 12. M. van Schie, J.D. Spöring, M. Bocola, P. Domínguez de María, and D. Rother, *Green*
364 *Chem.* **2021**, *23*, 3191-3206.
- 365 13. N. Guajardo, and P. Domínguez de María, *ChemCatChem* **2019**, *11*, 3128-3137.
- 366 14. P. Lozano (Ed), *Biocatalysis in green solvents*. **2022**. Elsevier, London, UK.
- 367 15. Y. Ni, D. Holtmann, and F. Hollmann, *ChemCatChem* **2014**, *6*, 930-943.
- 368 16. P. Domínguez de María, and F. Hollmann, *Front. Microbiol.* **2015**, *6*, 1257.
- 369 17. P. Domínguez de María, *Curr. Op. Green Sust. Chem.* **2021**, *31*, 100514.
- 370 18. M. Weiss, T. Brinkmann, and H. Gröger, *Green Chem.* **2010**, *12*, 1580-1588.
- 371 19. M. Paravidino, and U. Hanefeld, *Green Chem.* **2011**, *13*, 2651-2657.
- 372 20. S.K. Ma, J. Gruber, C. Davis, L. Newman, D. Gray, A. Wang, J. Grate, G.W. Huisman,
373 and R.A. Sheldon, *Green Chem.* **2010**, *12*, 81-86.
- 374 21. G.T. Höfler, A. But, S.H.H. Younes, R. Weber, C.E. Paul, I.W.C.E. Arends, and F.
375 Hollmann, *ACS Sust. Chem. Eng.* **2020**, *8*, 2602-2607.
- 376 22. D. Kuhn, M.A. Kholiq, E. Heinzle, B. Bühler, and A. Schmid, *Green Chem.* **2010**, *12*,
377 815-827.
- 378 23. S. Velasco-Lozano, E.S. da Silva, J. Llop, and F. López-Gallego, *ChemBioChem* **2018**,
379 *19*, 395-403.

- 380 24. U. Onken, A. Koettgen, H. Scheidat, P. Schuepp, and F. Gallou, *Chimia* **2019**, *73*, 730-
381 736.
- 382 25. G. Pigulewski, and M. Charik, *Biochem. Zeitschr.* **1928**, *200*, 201-210.
- 383 26. L.L. Wallen, R.G. Benedict, and R.W. Jackson, *Arch. Biochem. Biophys.* **1962**, *99*, 249-
384 253.
- 385 27. L.E. Bevers, M.W.H. Pinkse, P.D.E.M. Verhaert, and W.R. Hagen, *J. Bacteriol.* **2009**,
386 *191*, 5010-5012.
- 387 28. Y.X. Wu, J. Pan, H.L. Yu, and J.H. Xu, *J. Biotechnol.* **2019**, *306S*, 100008.
- 388 29. E.Y. Jeon, J.H. Lee, K.M. Yang, Y.C. Joo, D.K. Oh, and J.B. Park, *Process Biochem.*
389 **2012**, *47*, 941-947.
- 390 30. Y.C. Joo, E.S. Seo, Y.S. Kim, K.R. Kim, J.B. Park, J.B. and D.K. Oh, *J. Biotechnol.*
391 **2012**, *158*, 17-23.
- 392 31. J. Löwe, and H. Gröger, *Catalysts* **2020**, *10*, 287.
- 393 32. P.L. Hagedoorn, F. Hollmann, and U. Hanefeld, *Appl. Microbiol. Biotechnol.* **2021**, *105*,
394 6159-6172.
- 395 33. S. Prem, C.P.O. Helmer, N. Dimos, S. Himpich, T. Brück, D. Garbe, and B. Loll, *Microb.*
396 *Cell. Fact.* **2022**, *9*, 58.
- 397 34. For instance, the impact of enzyme production may become relevant, in environmental
398 terms, and should be considered in more holistic assessments. See, for instance: M. Becker,
399 S. Lütz, K. Rosenthal, *Molecules* **2021**, *26*, 573.

400 35. Electricity costs of the process may be relevant, but for laboratory assessments are not
401 considered. See reference 9, on the E⁺-Factor.

402 36. A. Castagna, D. De Simeis, E.E. Ferrandi, S. Marzorati, D. Monti, S. Serra, and M.
403 Valentino, *Catalysts* **2020**, *10*, 1122.

404 37. Q.F. Sun, Y.C. Zheng, Q. Chen, J.H. Xu, and J. Pan, *Biochem. Biophys. Res. Comm.*
405 **2021**, *537*, 64-70.

406 38. S. Serra, D. De Simeis, S. Marzorati, and M. Valentino, *Catalysts* **2021**, *11*, 1051.

407 39. P. Domínguez de María (Ed), *Industrial Biorenewables, a practical viewpoint*. **2016**.
408 John Wiley & Sons, Hoboken – NJ, US.

409 40. C.J. Clarke, W.C. Tu, O. Levers, A. Bröhl, and J.P. Hallett, *Chem. Rev.* **2018**, *118*, 747-
410 800.

411 41. R.K. Henderson, C. Jiménez-González, D.J.C. Constable, S.R. Alston, G.G.A. Inglis, G.
412 Fisher, J. Sherwood, S.P. Blinks, and A.D. Curzons, *Green Chem.* **2011**, *13*, 854-862.

413 42. D. Prat, J. Hayler, and A. Wells, *Green Chem.* **2014**, *16*, 4546-4551.

414 43. C.M. Alder, J.D. Hayler, R.K. Henderson, A.M. Redman, L. Shukla, L.E. Shuster, and
415 H.F. Sneddon, *Green Chem.* **2016**, *18*, 3879-3890.

416 44. D. Prat, O. Pardigon, H.W. Flemming, S. Letestu, V. Ducandas, P. Isnard, E. Guntrum,
417 T. Senac, S. Ruisseau, P. Cruciani, and P. Hosek, *Org. Proc. Res. Dev.* **2013**, *17*, 1517-1525.

418 45. M. Takeuchi, S. Kishino, S.B. Park, A. Hirata, N. Kitamura, A. Saika, and J. Ogawa, *J.*
419 *Appl. Microbiol.* **2016**, *120*, 1282-1288.

420 46. D. Holtmann, and F. Hollmann, *Mol. Catal.* **2022**, 517, 112035.

421 47. S. Leuchs, S. Na'ammieh, and L. Greiner, *Green Chem.* **2013**, 15, 167-176.

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