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Energising the E-factor: The E^+-factor

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ABSTRACT

The E-factor has become an important measure for the environmental impact of (bio)chemical reactions. However, summing up the obvious wastes generated in the laboratory neglects energy-related wastes (mostly greenhouse gases) which are generated elsewhere. To estimate these wastes, we propose to extend the E-factor by an energy-term (E^+-factor). At the example of a lab-scale enzyme fermentation, we demonstrate that the E^+-factor can constitute a multiple of the classical E-factor and therefore must not be neglected striving for a holistic estimation of the environmental impact.

1. Introduction

For more than 25 years now, Sheldon's E-factor has been an inspiration for researchers aiming at environmentally more acceptable chemistry [1,2]. The E-factor (E for environmental) provides a very simple, yet reliable measure to estimate the resource intensity of a given process or reaction and the wastes generated. Other mass-based environmental metrics such as process mass intensity (PMI) or reaction mass efficiency (RME) have not reached the same wide importance [2] and are mostly used in special industries such as small-molecule pharma [1].

The E-factor essentially sums up the wastes generated in the process including reagents, solvents (except water) and reaction aids (such as filter- and column materials) and puts them into relation to the amount of product generated (eq. (1)). Hence, the E-factor can be determined very easily from information found in lab journals or standard operation procedures.

$$E = \frac{\sum m(wastes)}{m(product)} \left[\frac{kg}{kg}\right] \quad (1)$$

Eq. (1). Sheldon's E-factor.

Despite its large success, the 'classical' E-factor exhibits a range of shortcomings. For example, the quality (in terms of environmental hazard or depleting resources) of a given waste component is not reflected by the E-factor. In principle, this can be compensated by the 'environmental quotient' (Q) taking the 'environmental unfriendliness' of a given waste into account. There is also an ongoing debate whether water should be included in the E-factor or not [1]. The concept of the complete E-factor (cEF) has been developed to enable the inclusion of water in E-factor calculations [1,3].

Yet, another limitation is that the 'classical' E-factor does not take the energy demands (heating, cooling, stirring, pumping etc.) of a reaction into account. Especially if the reaction energy is derived from electrical power, this is easily overseen as electricity simply comes from a wall socket. Today's electricity, however, to a very significant part is still obtained from burning fossil fuels (gas, oil, coal) resulting in emission of CO_2 into the atmosphere. In the European Union for example, still roughly 50% of the electricity is obtained this way resulting in CO_2 emissions of 315 g per kWh in 2015. On OECD average (2015) this value was even 404 $g_{CO_2}\ _{kWh}{}^{-1}$ [4].

Most contributions reporting an E-factor analysis neglect this factor. We therefore became interested in estimating the contribution of this 'hidden' E-factor contribution caused by the electricity generation. For this, we define the E^+-factor comprising the

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classical E-factor plus CO_2-emissions caused by electricity generation (eq (2)).

$$E^+ = \frac{\sum m(wastes)}{m(products)}\frac{kg}{kg} + \frac{W \times CI}{m(product)}\left[\frac{kWh \times \frac{kg(CO_2)}{kWh}}{kg}\right] \quad (2)$$

Eq. (2). The E^+-factor. W = electrical power used; CI = carbon intensity, i.e. the local average CO_2 emissions caused for the generation of electricity.

To evaluate the impact of electricity-caused CO_2 emissions on the E^+-factor we decided to examine the electricity consumption of lab-scale enzyme production. In particular, we chose the recombinant expression of the unspecific peroxygenase from *Agrocybe aegerita* (r*Aae*UPO) [5]. Unspecific peroxygenases (UPOs, E.C. 1.11.2.1) are promising catalysts for selective oxyfunctionalisation reactions [6,7]. Despite the fact that the synthetic application of peroxygenases is still in its infancy [8] their potential has been demonstrated through many examples of hydroxylation of non-activated C–H-bonds [9–15] and further examples of (stereo)selective oxyfunctionalisation reactions [6,7,16] making them very promising alternatives to existing oxyfunctionalisation catalysts [17].

Peroxygenases, however, like all heme-dependent enzymes, exhibit a pronounced instability against H_2O_2 [18], which is generally solved by *in situ* generation of H_2O_2 through reductive activation of molecular oxygen [16,19]. Various approaches for the *in situ* generation of H_2O_2 have been developed in the past years covering chemical [20–23], enzymatic [24,25], electrochemical [26–32] and photochemical approaches [33–39].

For this study, we focussed on a recently described formate oxidase from *Aspergillus oryzae* (*Ao*FOx) [40–42]. *Ao*FOx-catalysed H_2O_2 generation is attractive for preparative-scale reactions as gaseous CO_2 represents the only stoichiometric side product leaving the reaction mixture and therefore not further complicating downstream processing. Overall, a bienzymatic cascade for the formate-driven, aerobic and stereospecific hydroxylation of ethyl benzene as a model substrate was envisioned (Scheme 1).

Both enzymes were produced by recombinant expression at 10L-scale using *Pichia pastoris* as expression host for r*Aae*UPO and *Escherichia coli* for *Ao*FOx (see SI for more details). To determine the 'classical' E-factor, we added up the masses of all agents used for the fermentation such as buffer and nutrients and divided them by the mass of enzyme obtained (Table 1).

At first sight, high E-factors in the range of 2000–4000 were determined for both enzymes as crude products. In other words, about 2000–4000 kg of wastes were generated per kg of the crude enzyme. These numbers were even higher when taking the water used for the fermentations into account (E-factors up to 50000). We believe that at least in this case water must not be neglected from the E-factor calculation because it leaves the fermentation in contaminated form which necessitates further (energy-intensive) processing.

Table 1
E– and E^+-factors determined for r*Aae*UPO and *Ao*FOx.

	E-factor[a] [kg kg^{-1}] x 10^{-3}	E^+-factor[b] [kg kg^{-1}] x 10^{-3}
crude r*Aae*UPO [c]	4.3 (15.7)	110.9
purified r*Aae*UPO [c]	18.5 (209.0)	566.8
crude *Ao*FOx [d]	2.8 (49.8)	99.7
purified *Ao*FOx [d]	4.3 (106.1)	157.8

[a] Input of all material and reagents per kg of produced enzyme. The input considering water is shown in brackets.
[b] The E^+-factor furthermore takes energy demand and the thereby resulting CO_2 emission during enzyme production and purification into account.
[c] Calculated based on 778 mg r*Aae*UPO after ultrafiltration or 295 mg r*Aae*UPO retained after purification.
[d] Calculated based on 285 mg *Ao*FOx retained after full purification.

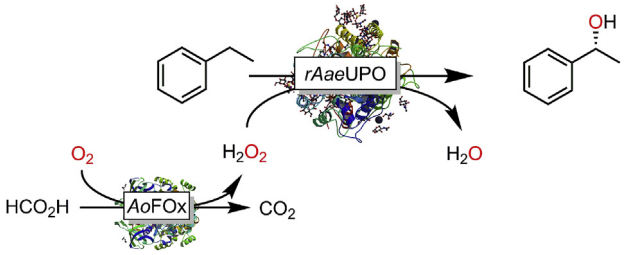
Table 1 also reveals the very significant contribution of electricity-related CO_2 emissions in the range of 100000 kg CO_2 per kg of enzyme.

Finally, also the contribution of enzyme purification is worth mentioning here. Depending on the number of purification steps and the solvent consumption for chromatographic purification steps, both the 'classical' E-factor as well as the E^+-factor were at least doubled (Table 1).

The biocatalysts themselves, however, are not the final products but only the catalysts for the reaction of interest. We therefore performed the desired stereospecific hydroxylation of ethyl benzene (Fig. 1).

Considering the high E^+- and cost-contributions of chromatographic protein purification we evaluated crude preparations as well as the purified preparations. While the time-courses for crude and purified r*Aae*UPO were essentially superimposable, no product formation was detectable using crude *Ao*FOx (Fig. 1). This was due to the presence of catalase in the crude *E. coli* preparations counteracting the *Ao*FOx-catalysed H_2O_2 generation. In case of r*Aae*UPO, being an extracellular easily sectreted enzyme, no catalase activity was detected in the crude preparations. Therefore, for all further calculations we used the E^+-factors of the crude enzyme for r*Aae*UPO and the purified *Ao*FOx, respectively (Table 1).

Table 2 compares the E-factor contributions of the enzymes for the preparation of (*R*)-1-phenyl ethanol as shown in Fig. 1. It should



Scheme 1. Bienzymatic cascade for the selective hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol. Formate oxidase (*Ao*FOx) mediates the formate-driven *in situ* H_2O_2 generation. The latter is used by the peroxygenase (r*Aae*UPO) for the selective hydroxylation reaction.

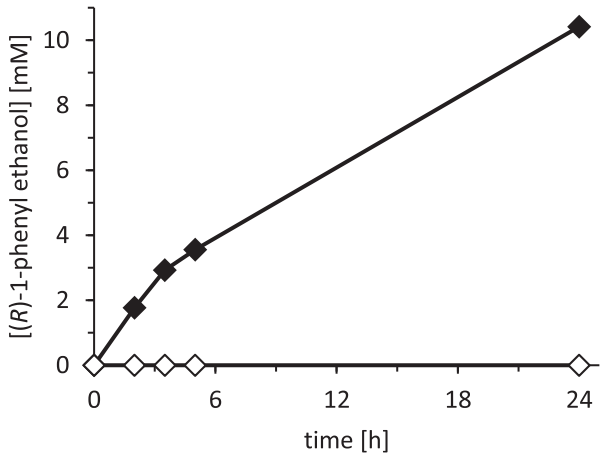


Fig. 1. Representative time course of the bienzymatic hydroxylation of ethyl benzene (Scheme 1) using purified (◆) or crude (◇) *Ao*FOx preparations. Conditions: KPi buffer (100 mM, pH 6), [r*Aae*UPO] = 200 nM, [*Ao*FOx] = 20 nM, [ethyl benzene] = 10 mM, [$NaHCO_2$] = 100 mM, T = 30 °C.

Table 2
E-factor evaluation of the reaction shown in Fig. 1.

Component	E-factor contribution [kg $kg^{-1}_{(R)\text{-1-phenyl ethanol}}$][a]
water	788
buffer[b]	7.7
r*Aae*UPO	7.1 (780141)[c]
*Ao*FOx	0.1 (15937)[c]
other[d]	<10
Sum	**813 (796884)[c]**

[a] a classical E-factor was calculated.
[b] Calculated as H_3PO_4.
[c] Numbers in parentheses take the E^+-factors for the enzymes into account.
[d] Including CO_2 from formate oxidation, non-reacted ethyl benzene, $NaHCO_2$ etc.

be noted that the reactions shown in Fig. 1 were performed on 1 mL-scale in a thermoshaker reaction setup and thus, we refrained from measuring the electricity used for the shaker; therefore Table 2 shows the E-factor calculation only. Based on mass, water is by far the biggest contributor to the environmental impact of the reaction. Also the contribution of the phosphate buffer (7.7 kg $kg^{-1}_{product}$) should not be ignored considering the fact that phosphate is a depleting resource. It should, however, be noted that the product concentration in this experiment (ca. 10 mM) was very low accounting for the high E-factor contributions of water and buffer [43]. Table 2 also illustrates the importance of taking the prehistory of the reagents into account. Performing the classical E-factor analysis just taking into account the actual masses of the biocatalysts used gives acceptable to excellent E-factor contributions for both enzymes. A completely different picture, however, evolves if the waste generation (mostly CO_2) during the enzyme preparation is taken into account. Similar numbers would most likely be the result taking the prehistory of the other reagents (substrate, buffer etc.) into account [44].

The numbers shown in Table 2 are prohibitively high to label the reaction shown in Fig. 1 as 'green'. Particularly the contributions of the biocatalysts are in stark contrast to the general notion that biocatalysis is a green technology. It should however be noted here that under the non-optimised reaction conditions the catalytic potential of the biocatalysts was by far not exploited yet. In the example shown in Fig. 1, r*Aae*UPO performed only 50000 catalytic turnovers and thereby fell back by orders of magnitude behind its catalytic potential [24]. *Ao*FOx performed 500000 catalytic turnovers.

The aim of this study was to evaluate to which extend 'hidden' waste formation originating from electricity generation contributes to the environmental impact of (bio)chemical processes. The numbers presented here clearly demonstrate that biocatalytic reactions by no means can be considered to be 'green' or 'environmentally benign' *per se* [45]. This impression may arise considering the 'classical' E-factor only while neglecting CO_2 emissions caused by the electrical power used. However, when taking this waste factor into account, a completely different picture evolves questioning the general notion of biocatalysis being an intrinsically green technology. However, we believe that these numbers can serve as a guiding principle to reduce the environmental impact of the reaction. As discussed below, already a few improvements can be very effective *en route* to this goal.

1.1. Enzyme preparation

As shown in this contribution, it takes more than just the obvious ingredients such as buffer and cultivation media to make an enzyme. For both enzymes the electricity-caused CO_2 emissions contributed to more than 80% of the total wastes generated. It should be noted that the numbers shown in Table 1 represent 'worst case scenarios'. r*Aae*UPO was produced in *P. pastoris* using a two-week fermentation protocol explaining the very high energy demand for its production. *Ao*FOx was produced from *E. coli*. Its overexpression protocol, however, is still far from being optimised. As a result, overall enzyme titres of approx. 22 mg L^{-1} fall far back from what can be achieved using *E. coli* as expression system and explain the high E^+-factor of *Ao*FOx. Considering that protein yields of up to several tens of grams of protein per litre fermentation broth (>10 g L^{-1}) can be achieved with *E. coli,* [46] an E^+-factor for an *E. coli*-borne enzyme in the range of 200–1000 kg kg^{-1} appears realistic; leading to acceptable overall contributions to the final product.

It should also be taken into account that the lab-scale fermentations reported here are rather small in volume. Industrial-scale fermentations in the m^3-scale may also profit from scaling-effects, more efficient energy usage and hence reduced E^+-factors [47,48].

Next to the fermentation itself, (chromatographic) purification also immensely adds to the environmental impact of an enzyme preparation. Consequently, from an economical point of view, purification is not attractive [49] which is also why the majority of industrial enzyme preparations are crude extracts rather than purified enzymes. In case of *Ao*FOx, some purification was inevitable to remove the competing catalase activity.

1.2. Reaction conditions

Obviously, the environmental impact of a catalyst directly correlates with its turnover number (TN). This is exemplified in Fig. 2. Increasing the TN of *Ao*FOx and r*Aae*UPO to approx. 1000000 and 3400000, respectively, reduces their E^+-factor contributions to the final product to 10 kg kg^{-1}.

Hence, the more efficiently an enzyme is used (in terms of TN), the lower its contribution to the overall waste generation.

Next to the biocatalyst contribution, water contributes significantly to the waste formation due to the poor solubility of the reagents. Increasing the reagent concentration is the most effective method to reduce this contribution, which can be achieved by neat reaction conditions [50] or using the two-liquid-phase system [36,43].

Overall, with this contribution we demonstrate that energy

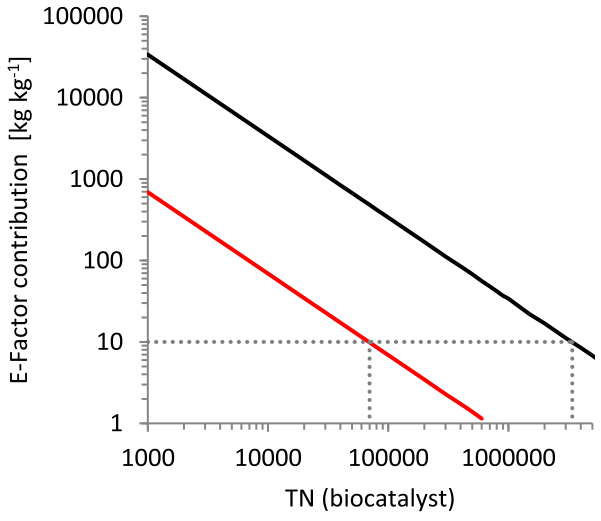


Fig. 2. E-factor contributions of r*Aae*UPO (—) and *Ao*FOx (-) to the final product depending on the turnover numbers achieved. The enzymes' E^+-factors shown in Table 1 put the basis for this calculation.

represents a factor that should not be neglected even from simple E-factor calculations. Enzymes are not *per se* 'green' catalysts and biocatalysis is not per *se a* 'green' technology. We all should critically reflect the possible environmental consequences of our reactions and processes before labelling them 'green' by default.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2019.01.065.

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