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Holistic Evaluation of Enzyme Immobilization Processes: A Method for Evaluating the Entire Production Process

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Enzyme immobilization plays a fundamental role in improving the industrial application of enzyme catalysis, as it greatly influences catalyst stability, reusability, and process control. However, due to the complexity of enzyme production and the variety of different immobilization strategies, research often focuses on isolated parts of the overall process, making an overall comparison of different production and immobilization strategies difficult. This study aims to present a structured, comprehensive method for the evaluation and comparison of immobilized enzyme processes. We identified 7 distinct process phases, each described by key performance indicators (KPIs), and showcase the evaluation in two case studies. In order to gain a complete insight, we then defined and calculated the meta KPIs "recovered activity efficiency", "space time activity", "total volumetric turnovers", and "total process productivity" by assessing the formally calculated KPIs for an efficiency-based evaluation approach. We also utilized the E Factor analysis as a sustainability-based evaluation approach to estimate environmental impacts. We showed that only a holistic view of all phases, by comparison of meta KPIs, allows for accurate comparison of the processes. Additionally, the structured evaluation can be used as a tool for the identification of weak points in each process to elucidate paths for improvement.

1. Introduction

Enzymes play an increasingly important role in today's economies as selective and sustainably producible catalysts. Compared to chemical catalysts, products are often purer, reactions require milder conditions and a lower catalyst loading is required.[1] Since the cost of enzymes is a main factor that limits the economic feasibility of enzyme-based production processes compared to chemical catalysis, enzyme immobilization has been and remains an important topic since the middle of the last century since it allows for repeated or continuous use of the enzyme, allowing for more catalytic turnovers per catalyst and thereby reducing the cost per turnover. Immobilized enzymes typically have a higher thermal, shear and pH stability making them a more robust catalyst. The immobilization itself not only enables continuous operation and reusability of the catalyst, but also offers several operational advantages. For example, packed-bed reactors simplify downstream processes; the enzymes are fixed in place, which facilitates a simplified separation of the product as well as the enzymes, improving process control and decreasing overall operation costs. Additionally, enzyme immobilization is necessary in some applications, like biosensors, to locate the catalyzed reaction for result reading or on a transducer.

Immobilization methods have generally been categorized into covalent and non-covalent binding on carriers, as well as immobilization by inclusion or carrier-free aggregation.[2] Carrier-based immobilization is commonly used and can utilize a variety of carrier materials and sizes, as well as surface properties linked to the immobilization mechanism. For example, amino- and epoxy carriers are used for covalent immobilization, amine carriers utilize surface charge for non-covalent ionic immobilization, and metal cation functionalized carriers are used for metal affinity immobilization. Depending on the application, either stable covalent immobilization with low leaching or the ability to recycle the carrier easily a reuse with a new enzyme (typically with noncovalent immobilization carriers) might be preferred. A recently emerging field is the in vivo enzyme immobilization, enabling one-step production and immobilization of enzymes. These immobilization methods include cell/phage surface display, inclusion body display, crystal-based protein entrapment, and virus-like particle display, among others, and can significantly reduce downstream processing time and cost, and carriers are usually biodegradable.[3] Downsides are a limited ambiguity to the immobilized protein for most methods and generally a small particle size, which can lead to issues related to enzyme retention. Detailed descriptions of the enzyme immobilisation methods can be found in several excellent reviews.[2,4–9]

The high industrial relevance of immobilized enzyme processes (IEP) is demonstrated by several examples of international companies producing immobilized enzymes as commercial products or using enzymatically catalyzed reactions with immobilized enzymes: phytases, peptidases, and lactases (Tolerase P, G, L; DSM), lipases (*Cal*B; Evonik), amylases/proteases

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(Lavergy; BASF), and D-amino acid oxidase and glutaryl amidase (Aventis). Phytases, peptidases, and lactases help digest phytic acid, gluten, and lactose, respectively, and are used as feed and food additives and food supplements. Immobilized *Cal*B (*Candida antarctica* lipase B) is used for applications like biodiesel production in >10,000 tons per year,[10,11] pharmaceutical synthesis[12] or food processing.[13] Amylases and proteases have a wide range of applications in the detergent industry. 7-Aminocephalosporanic acid is a precursor for cephalosporin, a beta-lactam antibiotic with a production of 1400 tons per year. It is produced in a two-step enzymatic cascade reaction using D-amino acid oxidase and glutaryl amidase.[14] In order to increase the number of industrially relevant enzymatic reactions, research into enzyme immobilization is one of the key factors in the transition from a chemical to a biotechnological industry.[15–18]

The nature of academic research and the complexity of the topic lead to partial descriptions of IEPs. Advancements are often made and reported in just one or a few phases from enzyme production, downstream processing of enzyme production (DSP1), enzyme immobilization, downstream processing of the immobilized enzyme (DSP2) or product formulation, which leads to biased assessment of the results and makes it almost impossible to compare different IEPs. Therefore, a meaningful comparison of IEPs has to take the entire process into account. However, for a true evaluation, it is necessary to consider not only individual steps but the entire IEP. For example, genetically engineered enzymes are often produced for targeted immobilization. The immobilization efficiency is then (significantly) improved, but the enzyme expression rate is (much) lower. Therefore, the actual efficiency can only be considered over the entire process chain.

In this concept paper, we aim to demonstrate the importance of considering the whole production process when comparing enzyme immobilization methods and propose a scheme to follow in order to carry out a proper, holistic evaluation of IEPs. We present both an efficiency-based approach, which evaluates the process on the basis of key performance indicators over several phases, calculating the 4 defined meta KPIs "recovered activity efficiency" (RAE), "space time activity" (STA), "total volumetric turnovers" (TVT) and "total process productivity" (TPP), and a sustainability-based approach, which simplifies the environmental impact of the production of immobilized enzymes (E Factor, Environmental Factor). First, the KPIs in the different phases of enzyme production, purification, and immobilization are described, followed by the calculation of the meta KPIs.

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Luisa Gebele received her Bachelor of Engineering degree in biotechnology at Offenburg University of Applied Sciences in 2023 after exploring the recombinant expression and biochemical characterization of fungal endochitinases in Komagataella phaffii. She is currently completing her Master's in Bioengineering at the Karlsruhe Institute of Technology and supports the working group at the Institute of Electrobiotechnology under the direction of Prof. Dr.-Ing. Dirk Holtmann in the field of enzyme expression and purification until February 2025.

Dirk Holtmann completed his diploma in chemical engineering/biotechnology in 1999. He received his PhD at the Otto-von-Guericke University Magdeburg on the electrochemical measurement of microbial activities. Until September 2019, he was head of the Industrial Biotechnology Group at the DECHEMA Research Institute. After that, he was Professor for Intensification of Bioprocesses at THM (Gießen, Germany). In 2023, he became a full professor for Electrobiotechnology at the Karlsruhe Institute of Technology. His current research activities focus on biocatalysis, bioprocess engineering, and bioelectrochemical synthesis.

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Two case studies demonstrate the practical applicability of the holistic approach with varying data availability. We hope that this work will contribute to a better comparability of published immobilization methods and to improved communication between experts in different aspects of enzyme production and immobilization, as well as between academia and industry.

2. Phases of Immobilized Enzyme Processes

In order to evaluate an IEP, it is necessary to define the different phases/stages in the immobilization process chain. We have divided the entire enzyme production and immobilization process into 7 individual phases, each with specific parameters to describe the individual phase (Figure 1). Besides measures of time, mass, volume, and amount of substance, an important parameter in the evaluation of any enzymatic process is the enzymatic activity. Enzymatic activity is described by the SI unit "katal" (kat), which means the amount of enzyme that catalyzes 1 mol of turnovers per second. Since that amount of enzyme is usually very large, most studies, including this one, use the non-SI unit of "enzymatic units" (U), which describes the amount of enzyme that catalyzes 1 µmol of turnovers per min.

$$1\ \mathrm{U}\left[\frac{\mu\mathrm{mol}}{\mathrm{min}}\right] = 16.67 \times 10^{-9}\mathrm{kat}\left[\frac{\mathrm{mol}}{\mathrm{s}}\right] = \frac{n_P}{t} \tag{1}$$

2.1. Phase 1 | Enzyme Modification

Some immobilization methods rely on prior enzyme modification. Affinity tags (Hexahistidine-tag, Strep-tag, metal-binding peptides, etc.), which are fused to the enzyme, enable oriented immobilization on functionalized carriers. Amino acid exchanges, such as the introduction of cysteine residues, can allow covalent immobilization on gold surfaces or functionalized carriers due to disulfide bridges.[19–21] Another immobilization technique is surface display, where the target enzyme is displayed on the outer membrane of the host cell by fusion with an anchor protein.[22] These enzyme modifications can affect the activity of the enzyme by interfering with its tertiary structure. Therefore, the first phase, "Enzyme Modification", is described by measuring the change in specific activity (A in [U mg^{-1}]) between the modified enzyme and wildtype ($A_{spec.}$ WT versus $A_{spec.\ modified}$). If the wildtype enzyme is produced and later immobilized, disregard phase 1, as subsequent changes in specific activity are categorized into the following phases.

$$A_{spec.}\left[\frac{\mathrm{U}}{\mathrm{mg}}\right] = \frac{A_{vol.}\left[\frac{\mathrm{U}}{\mathrm{mL}}\right]}{\text{protein conc.}\left[\frac{\mathrm{mg}}{\mathrm{mL}}\right]} \tag{2}$$

2.2. Phase 2 | Upstream Processing

The second phase includes the upstream fermentation of the enzyme. The outcome of an enzyme production depends on the nature of the expression host, the production scale, as well as the vessel and the operation mode (batch, fed-batch, continuous). Parameters used to describe this phase are: space-time yield (STY) or productivity, product yield per substrate (P S^{-1}), product yield per biomass (P X^{-1}), units per liter of fermentation broth (U L^{-1}), amount of enzyme per fermentation broth (mg_E L^{-1}) and total enzyme units (U).

$$\mathrm{STY}\left[\frac{\mathrm{mg}}{\mathrm{L\cdot h}}\right] = \frac{\text{enzyme amount }[\mathrm{mg}]}{\text{fermentation volume }[\mathrm{L}] \times \text{fermentation time }[\mathrm{h}]} \tag{3}$$

$$\frac{P}{S}\left[\frac{\mathrm{mg}}{\mathrm{g}}\right] = \frac{\text{enzyme amount }[\mathrm{mg}]}{\text{substrate amount }[\mathrm{g}]} \tag{4}$$

$$\frac{P}{X}\left[\frac{\mathrm{mg}}{\mathrm{g}}\right] = \frac{\text{enzyme amount }[\mathrm{mg}]}{\text{biomass }[\mathrm{g}]} \tag{5}$$

2.3. Phase 3 | Harvest

The third step describes the harvesting of the enzyme. Cells are separated from the culture broth and, depending on the production (extracellular versus intracellular), the cells or culture broth are retained for further processing. The harvest phase is described by the amount of enzyme (mg_E recovered mass) and enzyme units (U_E recovered units) recovered. For some non-conventional immobilization methods, especially in vivo immobilization approaches, this phase might not be necessary to fully describe the IEP, since these methods combine enzyme production and immobilization into one step (see case study 2 for reference).

2.4. Phase 4 | Downstream Processing 1

For intracellularly produced enzymes, phase 4 comprises the cell lysis (French press, high-pressure homogenizer, ultrasound), concentration (filtration, diafiltration), and further purification steps (precipitation, chromatography). For extracellularly produced enzymes, cells are separated from the supernatant by centrifugation, and the supernatant is processed in a similar way to intracellular enzyme production (concentration and purification). In all these steps, the enzyme can be either lost or inactivated, so this stage is again evaluated by determining the amount of enzyme (mg_E recovered mass) and the enzyme units recovered (U_E recovered units).

2.5. Phase 5 | Immobilization

Immobilization takes place in phase 5, where the choice of immobilization method (e.g., adsorption, embedding, covalent binding) and support material, and the subsequent possible preparation of the carrier, influence the parameters. The immobilization yield Y_I is defined as the proportion of the used

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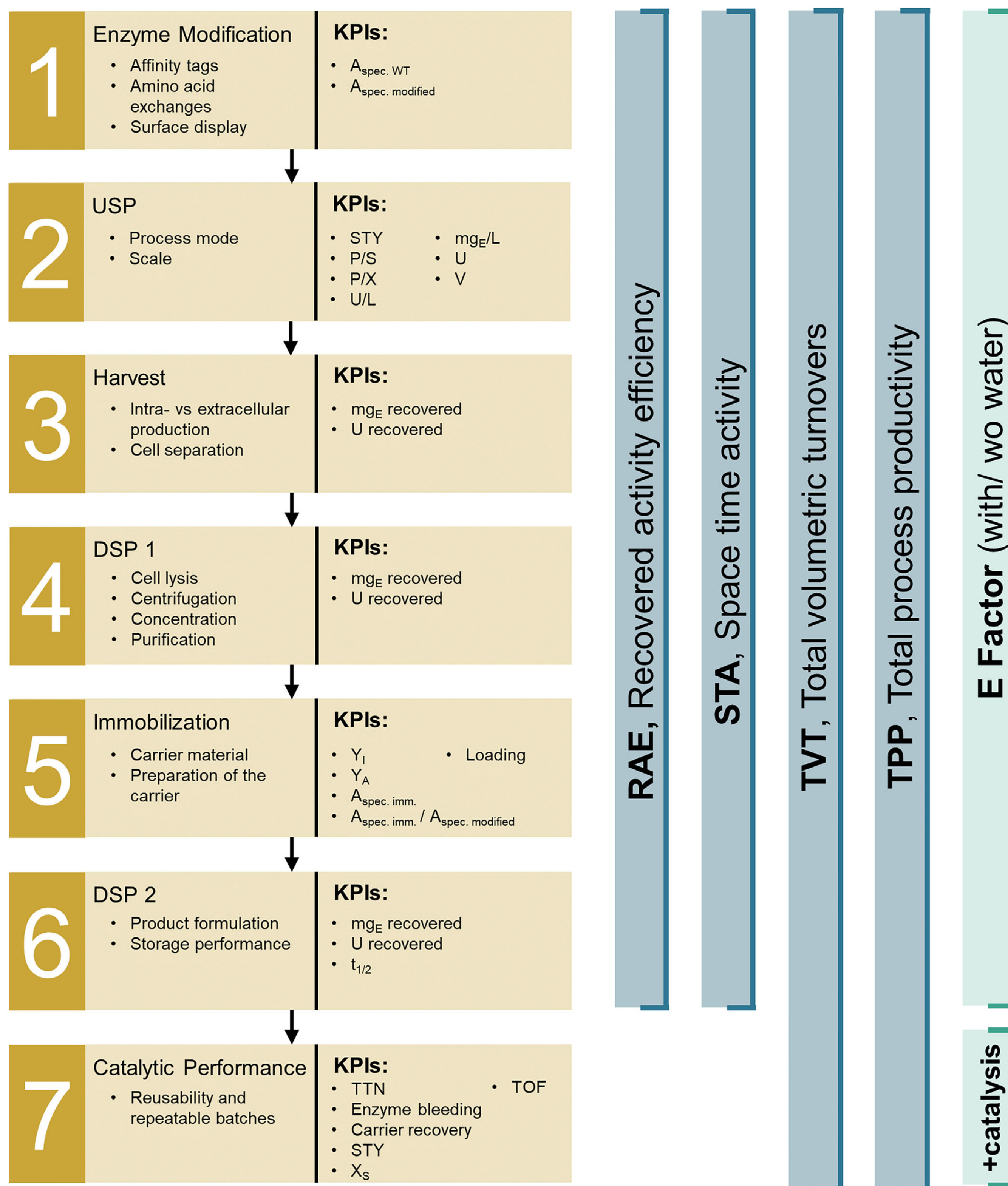

Figure 1. Phases of IEPs with factors influencing the KPIs describing each distinct phase and meta-KPIs describing the entire process.

enzyme (mg_E) that is immobilized on the carrier material. Alternatively, the activity yield Y_A is defined as the enzyme units found on the carrier after immobilization divided by the units of free enzyme used for the immobilization. The activity yield can exceed 100% in the case of an activation of the enzyme upon immobilization, but accurate determination requires a method of activity measurement for the immobilized enzyme. The $\Delta A_{spec.}$ describes the change in specific activity of the free enzyme

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compared to the immobilized enzyme. Additionally, the enzyme loading describes the enzyme units immobilized per mass of immobilization carrier.

$$Y_I\,[\%] = \frac{\text{immobilized enzyme }[\text{mg}]}{\text{used enzyme }[\text{mg}]} \times 100 = \left(1 - \frac{\text{non}-\text{immobilized enzyme }[\text{mg}]}{\text{used enzyme }[\text{mg}]}\right) \times 100 \quad (6)$$

$$Y_A\,[\%] = \frac{\text{immobilized enzyme }[\text{U}]}{\text{used enzyme }[\text{U}]} \times 100 \quad (7)$$

$$A_{\text{spec. immo}}\left[\frac{\text{U}}{\text{mg}}\right] = \frac{\text{immobilized enzyme }[\text{U}]}{\text{immobilized enzyme }[\text{mg}]} \quad (8)$$

$$\Delta A_{\text{spec.}}\,[\%] = \frac{A_{\text{spec.}}\left[\frac{\text{U}}{\text{mg}}\right]\text{ of immobilized enzyme}}{A_{\text{spec.}}\left[\frac{\text{U}}{\text{mg}}\right]\text{ of free enzyme}} \times 100 = \frac{Y_A\,[\%]}{Y_I\,[\%]} \times 100 \quad (9)$$

$$\text{Loading}\left[\frac{\text{U}}{\text{g}}\right] = \frac{\text{immobilized enzyme }[\text{U}]}{\text{used carrier }[\text{g}]} \quad (10)$$

2.6. Phase 6 | Downstream Processing 2

This phase is especially important if the immobilized enzyme is the end product of the described process and comprises product formulation as well as storage performance. Similar to phase 4, the key parameters are the amount of enzyme (mg_E recovered mass) and the enzyme units (U_E recovered units). Losses of both enzyme mass and activity can depend on storage and transport duration and conditions, residue in packaging, and others. The half-life ($t_{1/2}$) is defined as the period of time in which the activity of the enzyme is halved. k_d is the first-order rate constant determined by linear regression of ln(residual activity) versus the incubation time (t).[23] The half-life is used to describe the storage stability of the immobilized catalyst.

$$t_{1/2}\,[\text{h}] = \frac{\ln(2)}{k_d\ \left[\frac{1}{h}\right]} \quad (11)$$

2.7. Phase 7 | Catalytic Performance

To fully describe an immobilization method, the catalytic performance of the immobilized enzyme needs to be taken into account. The performance is influenced by stability of the enzyme on the carrier (enzyme bleeding), the possibility of retention or recovery of the enzyme and the reaction conditions (substrate, substrate and cosubstrate concentrations, temperature, pH, solvent concentrations, product concentrations, shear stress, diffusion limitations, etc.). The KPIs describing the catalytic performance are primarily the total turnover number (TTN), the conversion (X_S) and the turnover frequency (TOF; k_{cat}) of the enzyme in the given reaction. Under cost- and sustainability perspective it is desirable, to have the option of carrier recovery, once the immobilized enzymes have reached their catalytic capacity (TTN) and re-load them with fresh enzyme. This is often possible for non-covalent immobilization methods which in turn are more susceptible to enzyme bleeding (detachment of the enzyme from the carrier).

$$\text{TTN}\left[\frac{\text{mol}}{\text{mol}}\right] = \frac{\text{catalytic turnovers }[\text{mol}]}{n_{\text{biocat.}}\ [\text{mol}] \times \text{active sites per biocatalyst }[-]} \quad (12)$$

$$\text{TOF}\left[\frac{1}{\text{s}}\right] = k_{cat} = \frac{\text{catalytic turnovers }[\text{mol}]}{n_{\text{biocat.}}\ [\text{mol}] \times \text{active sites per biocatalyst }[-] \times t_{\text{catalysis}}\ [\text{s}]} \quad (13)$$

$$\text{Enzyme bleeding }[\%] = \frac{\text{amount of enzyme at } t_1\ [\text{mg}]}{\text{amount of enzyme at } t_0\ [\text{mg}]} \times 100 \quad (14)$$

$$X_S[\%] = \frac{n_{S,0}[\text{mol}]}{n_S\ [\text{mol}]} \times 100 \quad (15)$$

2.8. Meta KPIs

The thorough balancing of each process phase allows for the calculation of key performance indicators with a broad balance framework, describing the IEP in its entirety. These meta KPIs certainly lack the detailed information that the KPIs of each phase can deliver, but enable a concise, comprehensive comparison of multiple IEPs. We differentiate between efficiency-based meta KPIs and sustainability-based meta KPIs. Efficiency-based meta KPIs describe the units of immobilized enzyme per liter of fermentation volume (recovered activity efficiency, RAE, and space time activity, STA), either taking into account the time needed for enzyme production, DSP, and immobilization or not. Additionally, if the catalysis conditions are known, the catalytic turnovers per liter of fermentation volume (total volumetric turnovers, TVT, and total process productivity, TPP) can be determined, again taking into account the time component or not. Sustainability-based meta KPIs refer to the E Factor of the produced catalyst (phases 1–5, E $\text{Factor}_{\text{catalyst}}$) or the product of the catalyzed reaction (phases 1–7, E Factor), either taking into account water or not.[24,25] Water plays a highly important role in IEPs, particularly in the catalytic step. Not only is it used as the basis for fermentation media and buffers in the purification/immobilization

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process, but it is also often used as the main solvent in catalysis reactions. The water used throughout the entire IEP becomes contaminated in the end and cannot be reused without further energy-intensive treatment. Therefore, it must be included in the waste product calculation to accurately reflect resource utilization. However, water can often be recycled, and excluding it from the calculation allows for better comparison with chemical processes, in which solvents are often excluded.[25,26] To provide a comprehensive view of the process, we recommend reporting both values. Nevertheless, these numbers could be further improved by including the nature of the waste (toxic or nontoxic) and energy consumption in the calculations.[27,28]

$$\mathrm{RAE} = \frac{\mathrm{U_{immE}}}{\mathrm{L_{Ferm}}}\left[\frac{\mathrm{U}}{\mathrm{L}}\right] = \frac{\text{units of immobilized enzyme [U]}}{\text{fermentation volume[L]}} \tag{16}$$

$$\mathrm{STA} = \frac{U_{\mathrm{immE}}}{L_{\mathrm{Ferm}} \times d_{\mathrm{Ferm}}}\left[\frac{\mathrm{U}}{\mathrm{L} * \mathrm{d}}\right] = \frac{\text{units of immobilized enzyme [U]}}{\text{fermentation volume [L]} \times \text{fermentation time [d]}} \tag{17}$$

$$\mathrm{TVT} = \frac{\mathrm{TON_{immE}}}{L_{\mathrm{Ferm}}}\left[\frac{\mathrm{mol}}{\mathrm{L}}\right] = \frac{\text{catalytic turnovers [mol]}}{\text{fermentation volume [L]}} \tag{18}$$

$$\mathrm{TPP} = \frac{\mathrm{TON_{immE}}}{L_{\mathrm{Ferm}} \times d_{\mathrm{Ferm}}}\left[\frac{\mathrm{mol}}{\mathrm{L} * \mathrm{d}}\right] = \frac{\text{catalytic turnovers [mol]}}{\text{fermentation volume [L]} \times \text{fermentation time [d]}} \tag{19}$$

$$\text{E Factor}\left[\frac{\mathrm{g}}{\mathrm{g}}\right] = \frac{m_{\text{raw material}}\,[\mathrm{g}] + m_{\text{reagents}}\,[\mathrm{g}] + m_{\text{solvents}}\,[\mathrm{g}] - m_{\text{product}}\,[g]}{m_{\text{product}}\,[\mathrm{g}]} \tag{20}$$

3. Case Studies

In this section, we showcase the division of IEPs into the 7 process phases, the extraction of KPIs from literature, the calculation of missing KPIs, and the determination of meta KPIs from the collected and calculated data. The methodology is designed to be broadly applicable, so we showcase it for two different case studies: the first case study compares two processes with alcohol dehydrogenases (ADHs), a widely known and well-researched enzyme class. We assume that the immobilized enzyme is the end product in this case study (phases 1–6) and chose two very similar scales and immobilization approaches. Conversely, case study 2 compares processes with unspecific peroxygenases (UPOs), a relatively new enzyme class with not much data availability compared to ADHs. The compared processes include phases 1–7 and greatly diverge in the immobilization approach and scale. Furthermore, in phase 7, two different reactions are catalyzed. The chosen comparisons are intended to highlight the variety of processes to which this methodology can be applied and might therefore compromise the value that is gained by the specific comparison.

3.1. Case Study 1 | Alcohol Dehydrogenases

Industrially used medium-chain alcohol dehydrogenases (ADHs, EC 1.1.1.1) are zinc-containing enzymes belonging to the group of oxidoreductases. These enzymes catalyze the reversible oxidation of primary/ secondary/ cyclic alcohols to the corresponding ketone/ aldehydes using $NAD(P)^+$/NAD(P)H as cofactor. The enzyme is ubiquitous in bacterial, yeast, and animal cells. ADHs are divided into five distinct subgroups (ADH 1–5), of which ADH1 is the best known, catalyzing the oxidation and reduction of primary/ secondary alcohols and aldehydes to the corresponding ketones/ alcohols.[29–31]

Yeast ADH (YADH) from *Saccharomyces cerevisiae*, also known as baker's yeast, has several industrial applications in whole-cell catalysis (e.g., biofuel production) and biosensor technology (medical diagnostics of alcohols). However, the use of ADHs in the chemical and pharmaceutical industries for the synthesis of bulk chemicals and high-value chiral drugs is rare.[23,31–33] The need for a robust cofactor regeneration system and the high cost of enzyme production have limited its use on an industrial scale.[33]

In addition to oxidation reactions, ADH1 catalyzes the reduction of formaldehyde to methanol with high conversion efficiency.[34–36] This reaction is part of the three-stage enzymatic reduction cascade from CO_2 to methanol, which is a valuable way of recycling CO_2.

Since YADH-catalyzed reactions have been studied for more than half a century, a variety of different immobilization techniques and production systems (homologous/heterologous) have been reported. The two case studies we chose show the recombinant YADH production and subsequent immobilization on different carrier materials. The studies were selected because both provide sufficient key data for production and immobilization, allowing us to clearly demonstrate our method. Thus, we present a holistic comparison of the two IEPs by considering the entire enzyme production and immobilization process (Table 1).

Benito and coworkers published a study demonstrating the one-step purification and immobilization of a carbohydrate-binding module tagged YADH.[37] The affinity-tagged YADH is recombinantly produced in *E. coli* in a bench-scale reactor in fed-batch fermentation mode with a final fermentation volume of 2 L. Carbohydrate-binding modules (CBMs) are purification tags with high affinity for a wide range of polysaccharides. In the study, these CBMs are used for the direct immobilization of the enzyme on cellulose supports from crude cell lysate. The second study presents a production and immobilization approach based on a His tagged YADH.[38] A His tag (histidine tag) is a short peptide sequence that has a high affinity for nickel or cobalt

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Table 1. Comparison of the production and subsequent immobilization processes for alcohol dehydrogenases from *S. cerevisiae* with a carbohydrate binding module[37] and a histidine tag (immobilization[38] and production[39]).

Phase	KPI	Symbol	CBM9-Tag (Benito)	His-Tag (Varga)
1 | Enzyme modification	Specific activity of wildtype enzyme	$A_{spec,\ WT}$	300 U mg^{-1}	300 U mg^{-1}
	Specific activity of modified enzyme	$A_{spec,\ modified}$	65.3 U mg^{-1}	480–680 U mg^{-1}
	Substrate used for specific activity determination	–	Ethanol	Ethanol
2 | Upstream processing	Space-time yield	STY	926 mg$_{Enzyme}$ L^{-1} d^{-1} a)	100–167 mg$_{Enzyme}$ L^{-1} d^{-1} a)
	Product yield per substrate	P/S	6.7 mg$_{YADH}$ g$_{Glc}$$^{-1}$ a)	36.5 mg$_{YADH}$/g$_{Glc}$ a) (LB medium supplemented with glucose)
	Product yield per biomass	P/X	36.6 mg$_{Enzyme}$ g$_{CDW}$$^{-1}$	–
	Units per liter of fermentation broth	U/L	116,000 U L^{-1}	72,000–170000 U L^{-1} a)
	Amount of enzyme per fermentation broth	mg$_{enzyme}$ L^{-1}	1780 mg L^{-1}	150–250 mg L^{-1}
		Total U	232,000 U a)	79,200–187000 U a)
	Fermentation volume	V	2 L	1.1 L
3 | Harvest	Recovered mass	mg$_E$	–	–
	Recovered units	U_E	–	–
4 | Downstream processing 1	Recovered mass	mg$_E$	3480 mg	73 mg
	Recovered units	U_E	227,244 U a)	35,040–49640 U a)
5 | Immobilization	Immobilization yield	Y_I	99.7%	99%
	Specific activity	$A_{spec.\ Immo.}$	64 U mg^{-1} a)	97–137 U mg^{-1} a)
	Specific activity change	$\Delta A_{spec.}$	98%a)	20.2% a)
	Activity yield	Y_A	97.7%	20%
	Enzyme loading	–	30 U mL$_{carrier}$$^{-1}$	2500–3400 U mL$_{carrier}$$^{-1}$ a)
6 | Downstream Processing 2	Recovered mass	mg$_E$	3470 mg a)	72.27 mg a)
	Recovered units	U_E	226,664 U a)	7008–9928 U a)
	Half-life	$t_{1/2}$	38.7 h	–
7 | Catalytic performance	Catalyzed reaction	–	–	–
	Total turnover number	TTN	–	–
	Turnover frequency	TOF/k_{cat}	–	–
	Enzyme bleeding	–	–	–
	Carrier recovery yes/no	–	Yes	Yes
	Space-time yield	STY	–	–
	Conversion	X_S	–	–
Meta KPIs	U$_{immE}$ L$_{Ferm}$$^{-1}$	RAE	113,332a)	6570a)
	U$_{immE}$ L$_{Ferm}$$^{-1}$ d$_{Ferm}$$^{-1}$	STA	45,332 a) b)	2628a) b)
	E Factor$_{catalyst}$	–	76a)	479a)
	E Factor$_{catalyst}$ (w/o water)	–	16 a)	16 a)
	Catalytic turnovers L$_{Ferm}$$^{-1}$	TVT	–	–
	Catalytic turnovers L$_{Ferm}$$^{-1}$ d$_{Ferm}$$^{-1}$	TPP	–	–
	E Factor	–	–	–
	E Factor (w/o water)	–	–	–

a) Parameters calculated from numbers given in the mentioned publications.
b) Assumption 60 h fermentation and immobilization.

ions and can be used for protein purification in immobilized metal affinity chromatography (IMAC) and for immobilization of enzymes on Ni^{2+} supports. The recombinant production of the enzyme is described by Levarski and coworkers, who worked in a bench-scale reactor in batch mode with a final fermentation volume of 1.1 L.[39]

Analysis of individual phases allows a holistic view of the entire IEP and the identification of bottleneck steps in the process. Comparing both IEPs indicates a clear difference in the total mass of enzyme produced in the upstream phase (phase 1). The CBM9-tagged enzyme has a 10-times higher STY compared to the His tagged YADH, hence the fermentation yields higher

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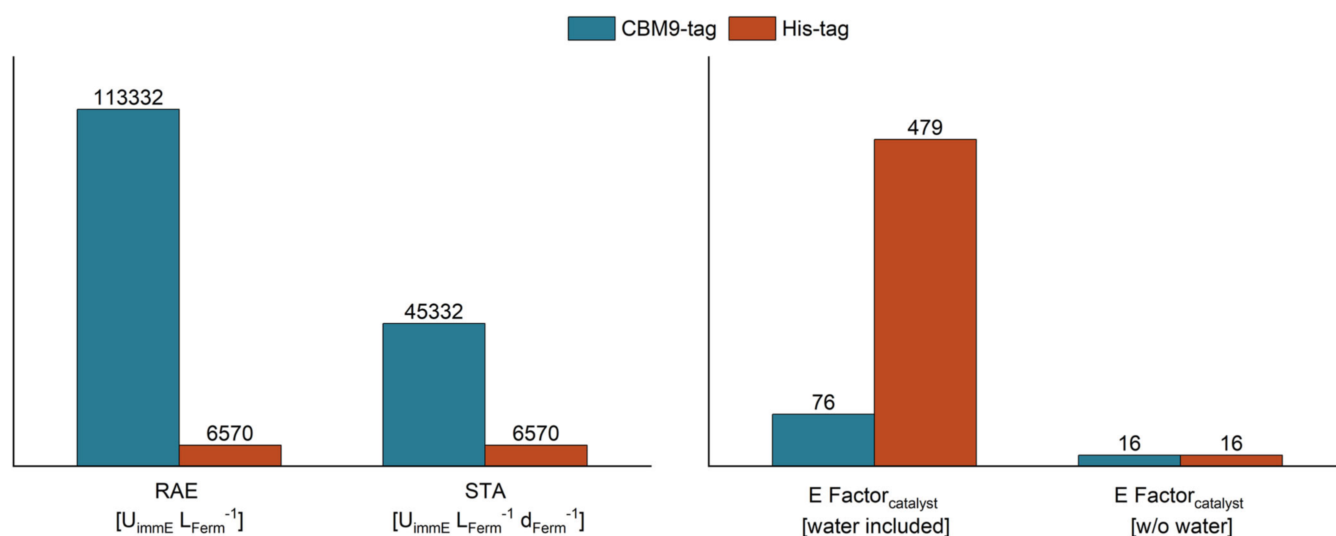

Figure 2. Comparison of the meta KPIs for the ADH case study. The meta KPIs RAE, STA, and E Factor$_{catalyst}$ are, by definition, calculated for phases 1–6.

enzyme titers. However, the units per liter of fermentation broth are in the same range for both upstream fermentations, so that both provide comparable amounts of active enzyme. This results from the higher initial specific activity of the His tagged mutant (480–680 U mg^{-1}), compared to the CBM9-tagged YADH (65.3 U mg^{-1}). For His tagged YADH, phases 4 (DSP 1) and 5 (immobilization) are the major contributors to the loss of enzyme units. In phase 4, less than half of the enzyme amount can be harvested from intracellular production, so more than half of the enzyme amount is lost due to insufficient enzyme extraction from the cells at this step. In phase 5, an activity yield of 20% is reported, which again contributes to the significant loss of enzyme units. A comparison of the two phases with CBM9-tagged YADH shows significant advantages over His tagged YADH. In contrast, a weakness of the CBM9 process is phase 1, where a lower initial specific activity is reported compared to His tagged YADH.

Comprehensive comparison of the two affinity tag-based methods is made by comparing the calculated meta KPIs (Figure 2). Both original papers did not provide data for the catalytic performance of the immobilized enzymes, so the meta KPIs are calculated for phases 1–5 for both YADHs (RAE and STA). For all calculations, active enzyme units or mass are treated as the product of the process. Although the His tagged mutant has a higher specific activity than the CBM9 tagged YADH, the entire process yields 20 times less the amount of active immobilized enzyme units per liter of fermentation broth (and time). Hence, the CBM9 process is more efficient regarding RAE. The higher activity yield and higher enzyme recovery from the cells lead to the increased efficiency-based meta KPIs. The sustainability-based meta KPIs also show an advantage for the CBM9 tag. The E Factor$_{catalyst}$ is six times lower than for the His tag process. Compared to the His tag, the CBM9 tag process eliminates further DSP steps after cell lysis, and the enzymes are immobilized directly from the crude lysate. This simplified DSP saves water and improves the E Factor.

3.2. Case Study 2 | Unspecific Peroxygenases

Unspecific peroxygenases are fungal heme-thiolate oxidoreductases that can catalyze the oxygenation of inert C-H bonds under ambient conditions using only hydrogen peroxide as a cosubstrate.[40]

Although the catalyzed reaction is industrially relevant and the alternative, petrochemical process routes often require high pressure and temperature while yielding non-selective products, industrial-scale use is not yet documented. One of the main reasons for this is certainly the high cost associated with enzyme production.[41] Therefore, it is imperative that the produced enzymes are used to their full extent, so the cost per turnover is reduced. Enzyme immobilization can often increase the turnovers per enzyme by stabilization effects and enabling process operation modes like repeated batch or continuous catalysis.[2]

Unfortunately, immobilization of UPOs is not yet an advanced technology, and most published methods show activity yields of 10% or lower.[42,43]

In this case study, we will compare different immobilization strategies for UPOs from enzyme production to catalysis performance in order to evaluate their readiness for application. By streamlining the evaluation of immobilized UPO processes, we hope to contribute to this research field and enable quick comparison to published methods. The selection of compared processes is determined mainly by the availability of data, which undermines the importance of considering the entire process and reporting data accordingly.

We recently published an immobilization strategy via yeast surface display[43] and compared its performance to a more traditional approach of immobilized enzyme production by production of free enzyme[44] and subsequent covalent immobilization via amino functionalization on a methacrylate carrier (Table 2).[45] The publication of Tonin and coworkers was used for phases 1–4 because i) it is the largest scale fermentation for

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Table 2. Comparison of IEPs for the unspecific peroxygenase from *Agrocybe aegerita*, variant PaDa-I, by production of free enzyme[44] and subsequent covalent immobilization,[45] and production of yeast-surface displayed enzyme.[43]

Phase	KPI	Symbol	Production of Free UPO & Subsequent Covalent Immobilization[44,45]	Yeast Surface Display UPO[43]
1 | Enzyme modification	Specific activity of wildtype enzyme	$A_{spec,\ WT}$	828 U mg^{-1}[46]	828 U mg^{-1}[46]
	Specific activity of modified enzyme	$A_{spec,\ modified}$	–	346 U mg^{-1}
	Substrate used for specific activity determination	–	ABTS	ABTS
2 | Upstream processing	Space-time yield	STY	63 mg_{Enzyme} L^{-1} d^{-1} a)	19.2 mg_{Enzyme} L^{-1} d^{-1} b)
	Product yield per substrate	P/S	1.18 mg_{Enzyme} $g_{Substrate(Glyc+MeOH)}^{-1}$ a)	0.38 mg_{Enzyme} $g_{Substrate(Glyc+MeOH)}^{-1}$ b)
	Product yield per biomass	P/X	2.9 mg_{Enzyme} g_{CDW}^{-1} a)	1 mg_{Enzyme} g_{CDW}^{-1} b)
	Units per liter of fermentation broth	U/L	298,000 U L^{-1}	51,000 U L^{-1} b)
	Amount of enzyme per fermentation broth	mg_{enzyme} L^{-1}	360 mg_{Enzyme} L^{-1} a)	147 mg_{Enzyme} L^{-1} a)
		Total U	738,000,000 U	204,000 U b)
	Fermentation volume	V	2484 L	4 L b)
3 | Harvest	Recovered mass	mg_E	294,000 mg_{Enzyme}	–
	Recovered units	U_E	294,700,000 U	–
4 | Downstream processing 1	Recovered mass	mg_E	170,000 mg_{Enzyme}	542 mg_{Enzyme} b)
	Recovered units	U_E	12,900,000 U	187,680 U b)
5 | Immobilization	Immobilization yield	Y_I	52%	92%
	Specific activity	$A_{spec.\ Immo.}$	13.75 U mg^{-1} a)	346 U mg^{-1}
	Specific activity change	$\Delta A_{spec.}$	1.70% a)	42%
	Activity yield	Y_A	1% a)	80%
	Enzyme loading	–	22 U $g_{ECR8315F\ (Purolite\ Ltd.,\ U.K.)}^{-1}$	29.5 ± 1.9 U g_{CDW}^{-1}
6 | Downstream processing 2	Recovered mass	mg_E	–	–
	Recovered units	U_E	–	–
	Half-life	$t_{1/2}$	–	–
7 | Catalytic performance	Catalyzed reaction	–	Ethyl benzene hydroxylation	Cyclohexane hydroxylation
	Total turnover number	TTN	909,000	357,000
	Turnover frequency	TOF/k_{cat}	5.55 s^{-1} a)	0.38 s^{-1}
	Enzyme bleeding	–	–	–
	Carrier recovery yes/no	–	No	No
	Space-time yield	STY	404 mg L^{-1} h^{-1}	9.56 ± 0.15 mg L^{-1} h^{-1}
	Conversion	X_S	6% a)	0.3% a)
Meta KPIs	U_{immE} L_{Ferm}^{-1}	RAE	486 U L^{-1} a)	45,900 U L^{-1} a)
	U_{immE} L_{Ferm}^{-1} d_{Ferm}^{-1}	STA	85 U L^{-1} d^{-1} a)	5960 U L^{-1} d^{-1} a)
	E $Factor_{catalyst}$	–	73,252 a)	7466 a)
	E $Factor_{catalyst}$ (w/o water)	–	12,724 a)	2663 a)
	Catalytic turnovers L_{Ferm}^{-1}	TVT	436 $mmol_{Product}$ L^{-1} a)	20 $mmol_{Product}$ L^{-1} a)
	Catalytic turnovers L_{Ferm}^{-1} d_{Ferm}^{-1}	TPP	76 $mmol_{Product}$ L^{-1} d^{-1} a)	3 $mmol_{Product}$ L^{-1} d^{-1} a)
	E Factor	–	104 a)	4235 a) (1397) c)
	E Factor (w/o water)	–	13 a)	396 a) (373) c)

a) Parameters calculated from numbers given in the mentioned publications.
b) Data from the 5 L bioreactor fermentation conducted for this study.
c) Values in brackets represent E Factor calculations for a single batch catalysis for 200 h instead of 10 batch catalyzes for 20 h each, assuming identical total product.

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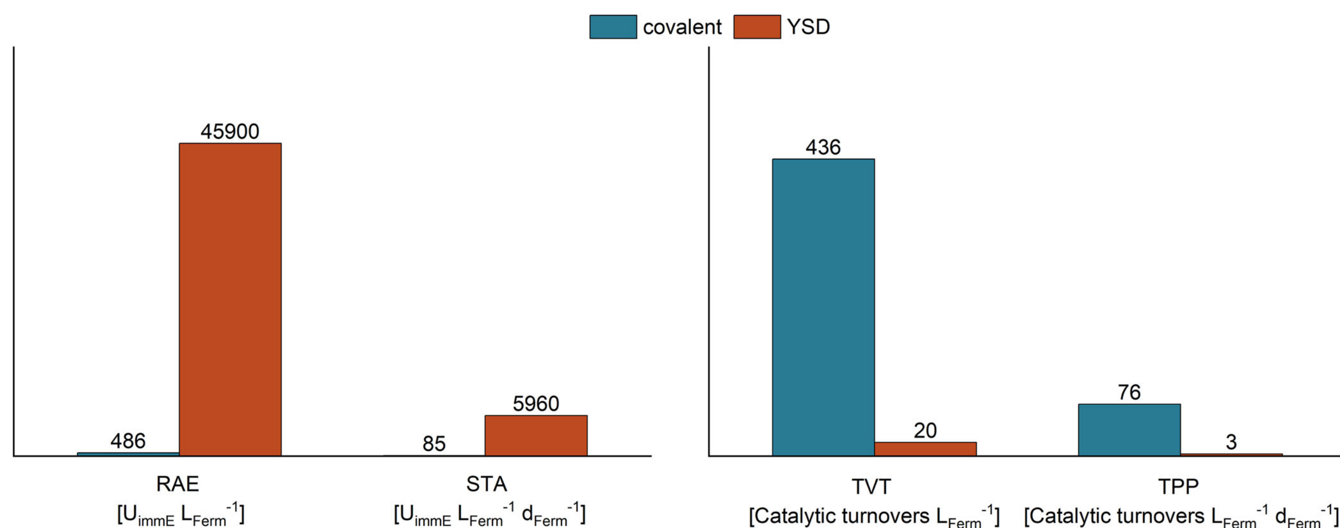

Figure 3. Comparison of the meta KPIs for the UPO case study. The meta KPIs RAE and STA are, by definition, calculated for phases 1–6. TVT and TPP are calculated for the entire production and immobilization process (phases 1–7).

the production of UPO that is reported in literature, and ii) the process parameters are well documented. For the phases 5 and 7 (phase 6 was neglected for this comparison) we refer to the study from Hobisch and coworkers because i) the reached total turnover number is the highest reported for the model UPO variant *Aae*UPO PaDaI and ii) again, the data for the immobilization and catalysis performance is well documented, allowing for calculation of the meta KPIs for the entire process.

For better comparability, YSD-UPOs were produced in a 5 L bioreactor with a protocol similar to that used in the fermentation of Tonin et al. and data of that fermentation (see supporting information) was used for evaluation of phase 2, instead of using the data from baffled shake flasks presented in the publication.[43]

Analysis of individual phases allows for the identification of weak points in the process chain. For the production of free UPO and subsequent immobilization, phase 5 is the main contributing factor to the loss of units. This is mainly due to the drastically reduced $A_{spec.\ Immo}$ after immobilization (1.7% of the free enzymes $A_{spec.\ WT}$), leading to an activity yield of only 1%. Additionally, phase 4 is also linked to substantial losses but can likely be easily improved, as discussed by the authors of the original study.[44] Phases 4 and 5 are also precisely the phases in which surface-displayed UPOs show obvious advantages compared to conventional immobilization processes, showing an activity yield of 80%. Contrary, the phases 2 and 7 can likely be substantially improved by determining optimized production parameters for YSD-UPO production and utilizing a more sophisticated catalysis setup for the hydroxylation reaction.

Comprehensive comparison of both processes can be done by evaluation of the meta KPIs (Figure 3). Although fermentation of the free UPO in a large scale shows better KPIs than fermentation of the YSD-UPOs in a 5 L lab-scale reactor and hydroxylation of ethyl benzene in a rotating bed reactor yielded higher TTN, STY and conversion than cyclohexane hydroxylation in a repeated batch catalysis with YSD-UPOs, the efficient immobilization strategy of surface displaying the UPOs and additionally simplifying the DSP leads to drastically increased meta KPIs compared to the "conventional" immobilization strategy of producing free enzyme and subsequent covalent immobilization on a carrier, if the catalysis is disregarded. The RAEs and STAs are in the area of two orders of magnitude increased for YSD-UPOs and sustainability-based meta KPIs are improved approximately by a factor of 10 and 5 (considering or disregarding water, respectively) if the immobilized enzyme is considered the product for the evaluation. When including the catalysis in the E Factor determination, using the amount of catalysis products, the conventionally immobilized UPO clearly outperforms the YSD-UPO. This is mainly due to the repeated batch setup of the YSD-UPO catalysis, that was designed to showcase reusability of the catalyst and not for catalysis performance and therefore had large excesses in substrate and buffer (or low catalyst loading), which contribute to the waste during E Factor determination. Assuming identical product amount without repeated batches (values in brackets in Table 2) to estimate performance in a single fed batch (same operation mode as in Hobisch et al.) improves the E Factor considering water significantly but only marginally if water is ignored for the calculation. However, the superior rotating bed reactor setup used with conventionally immobilized UPO still clearly outperforms the crude inverting 50 mL reaction tube setup used with YSD UPOs and the better conversion and TTN result in E Factors that are improved by factor 13–29 (considering or disregarding water, respectively). Similarly, catalytic turnovers per fermentation volume are better by a factor of 22–25 (TVT or TPP, respectively) for the conventional immobilization process due to the better catalysis performance of the rotating bed reactor.

Additional considerations for the comparison of both processes should include the catalyzed reactions and the implications of the immobilization matrix for potential use cases. Some of the meta KPIs are calculated using KPIs from phase 7, which is explicitly intentional to gain insight into the entire immobilized enzyme process (e.g., the process based on Tonin and Hobisch is evaluated far less environmentally friendly compared to the YSD-

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UPO process until considering phase 7), but can complicate the comparison if the catalyzed reaction is not identical, like in this case study. However, comparison of the production of different catalysis products with varying market values is important when determining the economic viability and sustainability of the process (see section 4). In such an economic evaluation, the cost of the immobilization materials has to be justified by the added value of immobilization, so a cheap immobilization method like yeast surface display might be preferred. Furthermore, the KPIs and meta KPIs are tools for the evaluation of efficiency and sustainability of the process, but disregard categorical parameters. For example, while the process based on YSD-UPOs might be desirable due to high efficiency-based meta KPIs, it might get ruled out for application in pharma- or food industry due to the immobilization matrix being genetically modified whole yeast cells.

4. Interpretation of Meta KPIs and Economic Evaluation

By following the methodology presented in this publication, a high information density table containing KPIs is created, which is then summarized by meta KPIs. The calculation of meta KPIs can be applied for a broad range of processes across different scales, industries, and reactor types, as they are "blind" to these parameters. Many phases can be skipped if they do not apply for the evaluated process (e.g., phases 3 and 4 are summarized in phase 4 for the yeast surface display of UPOs in case study 2, phase 6 is skipped when there is no storage step between immobilization and catalysis or phase 5 can be left out when evaluating a process without immobilization) while the validity of the meta KPIs is still maintained. An exception is processes that do not include phase 2, as all meta KPIs are referencing the fermentation volume. In these cases, either a free enzyme is bought, immobilized, and used for catalysis (phases 5–7) or an immobilized enzyme is bought and used for catalysis (phase 7), and the processes cannot be summarized via the proposed meta KPIs.

Although broadly applicable, the interpretation and importance of each meta KPI might vary depending on the evaluated process. From an efficiency standpoint, the TPP is certainly the most holistic meta KPI as it includes the important process phase 7 (catalysis) as well as a time component and should be employed as a decision-making metric if possible. The value of the catalysis product (per amount of substance) can be multiplied with the TPP and the process cost per hour and fermentation volume (operation cost; OpEx) has to be subsequently subtracted for a simple economic evaluation of the process.

$$\text{Profit}\left[\frac{\Delta}{L \cdot d}\right] = \text{TPP}\left[\frac{\text{mol catalytic turnovers}}{\text{L}\cdot\text{d}}\right] \times \text{value}_{\text{product}}\left[\frac{\Delta}{mol}\right] - \text{OpEx}\left[\frac{\Delta}{\text{L}\cdot\text{d}}\right] \quad (21)$$

A complete economic evaluation also includes capital investments, required to build the production site, divided by estimated lifetime (CapEx). For further guidance on the evaluation of biological process costs, we recommend the study of Tufvesson and coworkers, although the costs for the production of biocatalysts (especially on a large scale) have likely decreased for many catalysts since publication.[41] This economic evaluation is especially important when comparing processes of significantly different scales, products, or conventional versus non-conventional immobilization methods, as these parameters greatly influence the process costs per amount of product. If the immobilized enzyme is intended as the final product (immobilized lipase of *Candida antarctica* from Novozymes, Novozyme 435 is a commonly utilized example), the meta KPI STA is the equivalently useful metric to the TPP.[47] The meta KPIs RAE and TVT are similar to STA and TPP, respectively, but lack the time component and should therefore be used for decision making if the fermentation time is not the limiting factor in the process chain.

Depending on the industry, requirements for cost and enzyme purity might differ significantly (e.g., the pharma industry typically has high purity requirements while the chemical industry is more concerned with the cost of the DSP). The presented methodology focuses on process efficiency and does not list purity as a KPI but instead assumes that the evaluated DSP is sufficient for the required purity.

E Factor calculation is independent of the efficiency-related meta KPIs and serves as an estimation of the sustainability of the process. Like the other meta KPIs, the E factor is "blind" for scale, reactor types, type of product, or industry type, and therefore has to be interpreted in its context. Its importance and actual impact on sustainability are largely dependent on the type of produced waste, moral values of the producing business, and quantity of product, but the authors recommend performing the E Factor calculation as a standard procedure when evaluating IEPs, considering the importance of sustainability in today's society. The E Factor is linked to economic decision making via waste disposal costs as well as customer acceptance of products and potential political incentives/fines depending on sustainability.

5. Summary and Outlook

This concept paper presents a holistic methodology for the evaluation of immobilized enzyme processes (IEPs). By presenting two different case studies, we highlight the necessity to take into account not only single parts of the process, but present meta KPIs, which help to evaluate different processes on both an efficiency- and sustainability-based approach. The proposed method enables a detailed view of the different phases of enzyme production and immobilization processes and offers valuable insights into each different phase. Therefore, bottleneck steps can be identified more easily and addressed. The developed evaluation approach can serve as a tool for researchers not only to compare different methods but also to improve and assess IEPs for industrial applications. Using this method allows researchers to more easily identify weak points in their processes (KPIs), perform a comprehensive evaluation

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(meta KPIs), and assess the economic efficiency of the process by combining meta KPIs with a cost evaluation. Lastly, we emphasize that researchers to implement FAIR data management practices to ensure the reproducibility and accessibility of data generated in enzyme immobilization studies. We provide a standardized Excel format in the Supporting Information (S1), in which all KPIs and meta KPIs can be listed for each process. We emphasize the importance of including this table in future publications to ensure the publication of metadata in a standardized communication protocol. This standardized format could help apply advanced data analysis techniques to accelerate enzyme immobilization development and streamline experimental workflows.[48,49]

Supporting Information

The authors have cited additional references within the Supporting Information.

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Conflict of Interests

The authors declare no conflicts of interest.

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