



# Intensification of oxygen-dependent biotransformations catalyzed by immobilized enzymes

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Oxidative biotransformations find a prominent role in the fine chemical industry and the valorization of renewable feedstocks. Implementation of oxygen-dependent reactions faces some challenges across scales and at different levels of development. First, the fruitful development of enzyme candidates and identification of reaction possibilities is not in consonance with the implementation in process engineering. Second, reaction engineering faces a complex interplay of reaction kinetic, oxygen transfer and process stability. Third, given the advances in synergic fields such as molecular biology, chemistry, material sciences, and (micro)process engineering, an interdisciplinary assembly from a consistent discipline around heterogeneous biocatalyst engineering would be of strategic value. We show advances in design of active and robust immobilized enzyme catalysts to be applied in (continuous) intensified processes. A framework based on the joint design of a catalyst and reactor will be discussed for the design and optimization of the catalysts and biotransformations involved.

## Addresses

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## Introduction

Oxidations are one of the most important transformations in the chemical industry. Besides well-established transformations in pharmaceutical and fine chemistry, diverse oxidative transformations are fundamental reactions in the processing, valorization, and upgrading of renewable raw materials in integrated biorefineries [1–3]. Biooxidations are a great alternative

to chemocatalytic methods of performing oxidations that suffer from numerous limitations: harmful oxidants, side reactions, lack of regioselectivity and stereoselectivity, and so on. In addition, oxidative enzymes enable chemists to perform highly selective and efficient transformations; for many of them, no chemical counterpart is known [3,4]. Biological oxidation also allows the use of molecular oxygen as the oxidant, which allows mild condition efficiency in terms of atom economy [3].

Oxygen-dependent enzymatic oxidation is approaching maturity and practical applicability in synthesis at the laboratory scale [2–4]. From a mechanistic point of view, the reactions involve oxygenation and dehydrogenation. Therefore, the application of oxidative enzymes is interesting across different areas of the biochemical technology, from integrated biorefineries to pharmaceutical chemistry, and using different raw materials [1,11]. The portfolio covers the selective oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones catalyzed by copper or flavin-dependent oxidases acting on aliphatic alcohols or carbohydrates [5–7]. Another group is the laccases and multicopper oxidases which oxidize organic substrates with application in synthetic chemistry [8]. Mono-oxygenases catalyze the selective hydroxylation or the aerobic oxidation of ketones and cyclic ketones to esters and lactones, respectively (Baeyer–Villiger mono-oxygenases). Beyond these well-established transformations, the new waves of biocatalyst development constantly bring new reactions, for example, the oxidation of secondary alcohols, amino alcohols, and 5-hydroxymethylfurfural [9,10].

The plethora of chemical reactions that oxidative enzymes provide is not sufficiently aligned with the challenges at reaction engineering level [4,12]. In process engineering the aim is the achievement of a required intensity (reaction rate and product concentration and purity) and process stability [12,13], which are consequent with sustainability and green criteria. Diverse issues such as low enzymatic activity, catalyst instability, inhibitions, cofactor regeneration, and substrate supply jeopardize a suitable development across scales. In addition, the kinetic analysis and reaction implementation of oxygen-dependent biocatalysts encounter the thermodynamic and kinetic limitations of the sufficient oxygen supply. The low solubility of the oxygen not only limits its maximum possible supply rate but

also has a direct effect on the efficiency with which a given oxidation enzyme is used [12,13]. To armor the oxidative biocatalysis as enabling technology, different disciplines as protein engineering, catalyst engineering, and process engineering applied from earlier stages of research development offer a promising toolbox [14,15]. In particular, enzyme immobilization and heterogeneous catalysis are fundamental instrumental tools for biocatalysts implementation and intensification [16,17], Figure 1. Known advantages are the reuse, continuous use, facilitation of reactor design across scales, and simplification of downstream processing. In this review, we describe recent advances in the design and application of enzyme-immobilized oxidative catalysts. We focus the review on the need for an integrated chain development of oxidative enzymatic processes implemented since early stages of research, and it can be summarized in three levels involving two main steps of development that require focused attention on the effectiveness of catalyst performance and integration into the reactor, Figure 1.

### Development of oxidative enzyme catalysts: focusing on activity

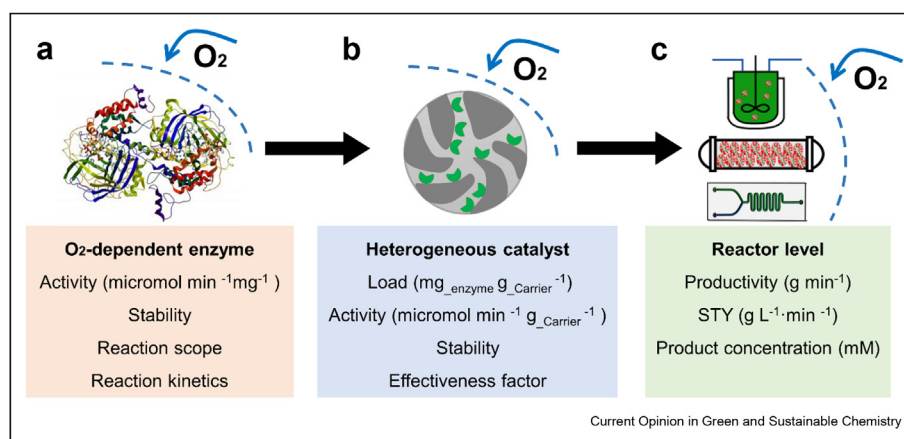
The successful implementation of oxidative enzymatic transformations depends on the availability of active, stable, and cost-efficient catalysts. Activity and stability of soluble catalysts can be engineered by conjoined strategies of protein engineering and molecular biotechnology [14,15,18]. Enzyme immobilization is an enabling technology for the design of oxidation biocatalysts. The implementation as solid-supported biocatalyst requires the selection and application of carrier material, immobilization chemistry, and suitable characterization under realistic operational conditions [13,14,19]. Ideally, the catalyst would display a high

activity upon a broad range of substrate concentration, and the activity would be kept under continuous operation or in several consecutive batches, Figure 2. The catalyst should be efficiently integrated in an enhanced reactor to guarantee a stable operation under intensified reaction conditions. This reaction output is a consequence of multiple decisions taken during the catalyst research and development, Figure 2.

The maximum reaction rate due to a certain amount of catalyst is the activity ( $\text{micromol}^{-1} \text{ min}^{-1} \text{ g}_{\text{solid}}^{-1}$ ), which depends on the amount of catalyst loaded into the material ( $\text{mg}_{\text{enzyme}} \text{ g}_{\text{solid}}^{-1}$ ) and on the specific activity of the immobilized enzyme ( $\text{micromol}^{-1} \text{ min}^{-1} \text{ mg}_{\text{enzyme}}^{-1}$ ). Loading increase is at the interface of suitable immobilization chemistry and structural features of the carrier materials [20–23]. Advances in material engineering and fine controlled enzyme immobilization allow pushing the current limits [20–23]. Usually, the specific activity of the immobilized enzyme differs from that of the free enzyme. Parameters to express that difference and quantify immobilization efficiency are plentiful, and harmonization is a need for effective scientific dissemination [13,18,19]. One reaction engineering parameter to address such variation is the effectiveness factor (catalytic effectiveness) that expresses the ratio of reaction rate provided by immobilized and free enzyme (expressed in the same amount of reference unit: e.g. mg of enzyme). Figure 3a shows a schematic design window of an immobilized biocatalyst depending on different underlying phenomena.

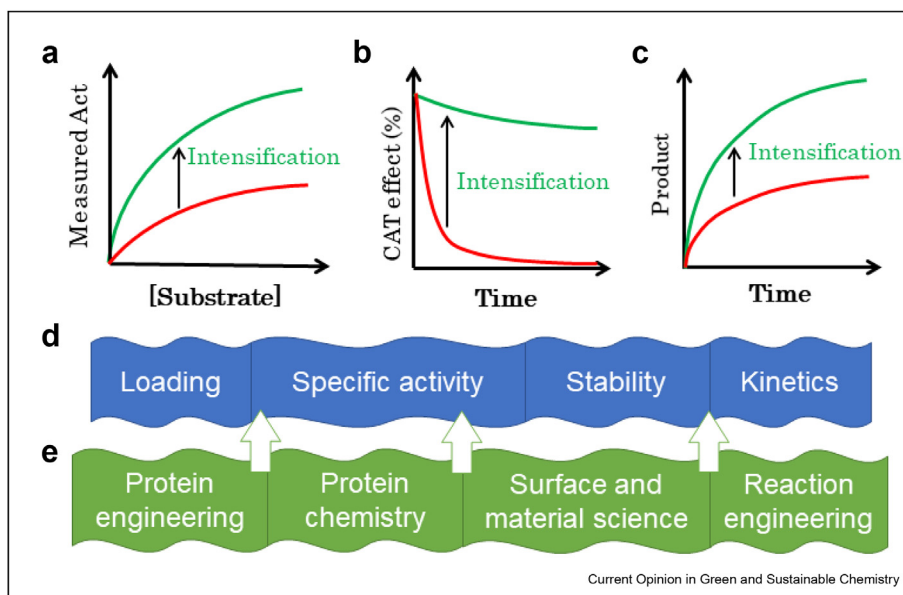
The effectiveness factor might be below one due to different reasons such as the structural distortions upon immobilization, also known immobilization effects, or physical phenomena as partition effects and mass transfer

Figure 1



**Chain of development of a solid-supported enzyme-catalyzed oxygen-dependent reaction.** (a) shows the free enzyme level and key parameters. Kinetics toward the main substrate and oxygen are of relevance to understand future bottlenecks. (b) shows the immobilized enzyme level and key parameters of design, which defines the activity and stability potential of the biocatalyst. (c) shows the reaction engineering level, where catalyst properties are translated in sufficient intensity and productivity.

Figure 2



**Characteristics of an intensified oxygen-dependent solid-supported enzyme catalyst.** The immobilization design must pursue excellent kinetics at stable conditions (high activity at broad range of substrate concentration), (a) excellent catalytic effectiveness (behavior of the immobilized enzyme is similar to that of the free enzyme regarding maximum velocity, that is kept over time (high stability, (b)). Therefore, product formation is boosted over time (c). The achievement of intensification is parametrized in a few key variables (d) which depend on the (ideally) holistic approach of different enabling technologies (e).

resistances, Figure 3b. In the map, we illustrate how, at low enzyme loadings, the values of measurable activity are normally a more direct response of the structural consequences of the immobilization, Figure 3a. The group of 'structural consequences of immobilization' is a diverse, normally black box that comprises multiple molecular levels. First, the activity of the immobilized oxidative catalyst must deal with redox cofactor functionality, which might require external supply, regeneration, or reactivation under reaction conditions [24,25]. Moreover, the efficient structural assembly of oxidases and monooxygenases is not a trivial task. In addition to the importance of enzyme orientation and minimal distortion of secondary and tertiary structure, the quaternary assembly of their multimeric or multidomain conformation is needed upon immobilization [6]. In this latter sense, oriented programmable immobilization through binding modules genetically fused has provided structural stabilization [26–28]. Another strategy is the use of postimmobilization strategies to ensure multiunit assembly and the fine control of mild covalent attachment [18,29].

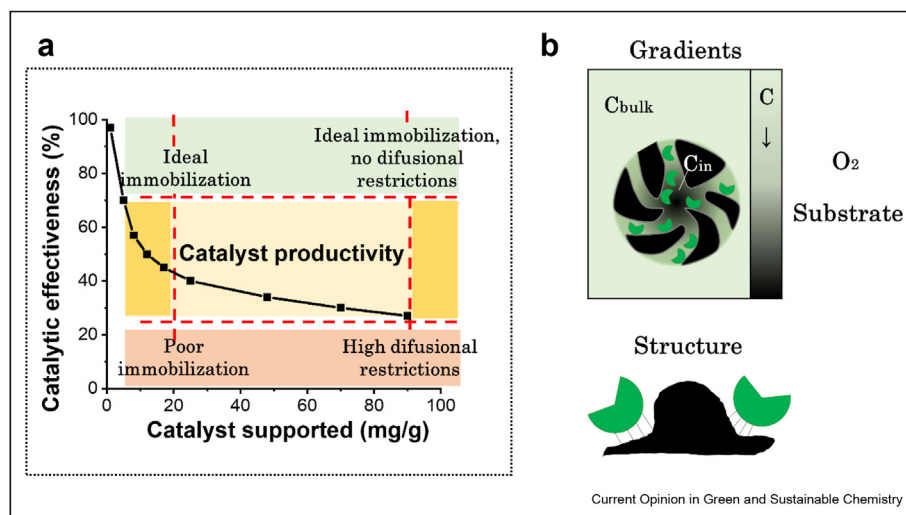
Values of observed activity vary in literature [16,30–32]. In Figure 3a, the importance of performing a complete plot of activity of immobilized enzyme catalysts to identify trends and underlying limiting phenomena is emphasized. At high activity (loading), the efficient supply of substrates, especially oxygen, into the solid

phase is one of the main challenges to maximize the activity of the immobilized enzymes [13,33]. Carrier physicochemical properties, carrier size, and catalyst localization are key design variables in the interplay with reaction kinetics. Enzyme loading and particle size critically influence the diffusional limitations and hence the measurable specific activity of the immobilized enzyme [34,35]. The study and design of the biocatalyst can be guided by classic chemical reaction engineering analysis or implemented in operando intraparticle sensing [13,33]. Measurement of the available  $O_2$  concentration inside solid carriers has provided direct evidence of the occurrence of diffusional restrictions undergone by  $O_2$ -dependent immobilized enzymes [34,35]. Recently, it was shown how the oxidase localization into the external shells of the particle is also a key parameter to minimizing the oxygen gradient and therefore mitigating the limitations of the oxygen supply to the immobilized enzyme [36,37].

### Development of oxidative enzyme catalysts: focusing on stability

Product formation along operation time depends on reaction kinetics [38–41], on how the velocity of each relevant reaction is affected by the concentrations of reaction components, and on the catalyst stability, Figure 2. Regarding the former, elucidation of kinetics of oxygen-dependent enzymes is not a trivial task. Recent developments from Lindeque and Woodley [38],

Figure 3



**Catalytic effectiveness of an oxygen-dependent solid-supported enzyme catalyst.** (a) shows the design window for the immobilization of an oxidase with the goal of maximizing catalyst productivity, which is the result of enzyme load and catalytic effectiveness. Constraints on the design are indicated with dotted lines, revealing major limitations due to enzyme loading and catalytic effectiveness. Boosting loading requires material design. Boosting catalytic effectiveness requires identification and removal of the overall limiting factor, which could be immobilization chemistry or mass transport. (b) shows the two main group of causes underlying the effectiveness factor decrease. (a) was adapted from the study by Bolivar et al. [20].

Toftgaard Pedersen et al [39], Meissner et al [40], and Ringborg et al [41] have shown advances dealing with reaction setup and deeper understanding between kinetics and process performance [38–41]. The stability of the catalyst should match the process requirements, and ideally, the analysis should be performed under realistic characterization at research level. The stabilization by design of the immobilized enzyme requires the identification of the inactivation controlling step. In homogeneous catalysis, enzyme inactivation by gas–liquid interfaces is a dominant phenomenon that can be solved when an enzyme is confined in mesoporous materials by protecting the enzyme from these interfaces [14]. Another characteristic inactivation route is caused, in oxidases, by hydrogen peroxide. This inactivation can be avoided or reduced by coupling an in-series reaction where the hydrogen peroxide is consumed or by coupling a parallel reaction to decompose hydrogen peroxide and produce oxygen as the one catalyzed by catalase [42–45]. In this last case, the instantaneous elimination of hydrogen peroxide can be achieved by co-immobilization of the oxidase and an auxiliary catalase inside the porous structure of solid support. Despite the high catalytic turnover of catalase loading and colocalization of the catalase are a major component of the stabilization [44,45], as recently shown by García-García et al [45], more than 93% of the hydrogen peroxide can be removed by coimmobilizing around 5–6 mg of catalase per mg of oxidase. Beyond modification of primary structure, enzyme inactivation comprises multiple effects of structural distortion. The

operational conditions for the stabilization of enzymes and their protection against denaturing agents are well described for multiple cases and represent a substantial body of the science of immobilization of enzymes [14,17,46]. Recent examples reflect the increasing tailor-made application to oxidative enzymes. Covalent immobilization of galactose oxidase, choline oxidase, and monoamine oxidase displayed long-term stability and reusability when covalently attached on a solid support, outperforming their free enzyme from both thermostability (more than 20-fold) and solvent stability [31]. Solé et al [28], García-Bofill et al [32], Delgove et al [47], and Solé et al [48] have developed different oxygen-dependent oxidative catalysts of industrial interest by immobilization engineering. For example, a eugenol oxidase was stabilized and immobilized, allowing the biocatalyst to be reused up to 18 reaction cycles and improving the biocatalyst productivity more than 12-fold [49]. In another example, a Baeyer-Villiger monooxygenase has been immobilized with a glucose dehydrogenase for the synthesis of  $\epsilon$ -caprolactone derivative and evaluated for several consecutive cycles. Coimmobilization proved to provide the most efficient approach with an average conversion of 83% over 15 reutilization cycles leading to a 50-fold increase of the biocatalyst productivity compared with soluble enzymes when applying a fed-batch strategy [47]. When the molecular stability of the free enzyme is sufficient, reversible directed immobilization or mild covalent attachment are useful tools to ensure a suitable activity–stability compromise [24,29,50].

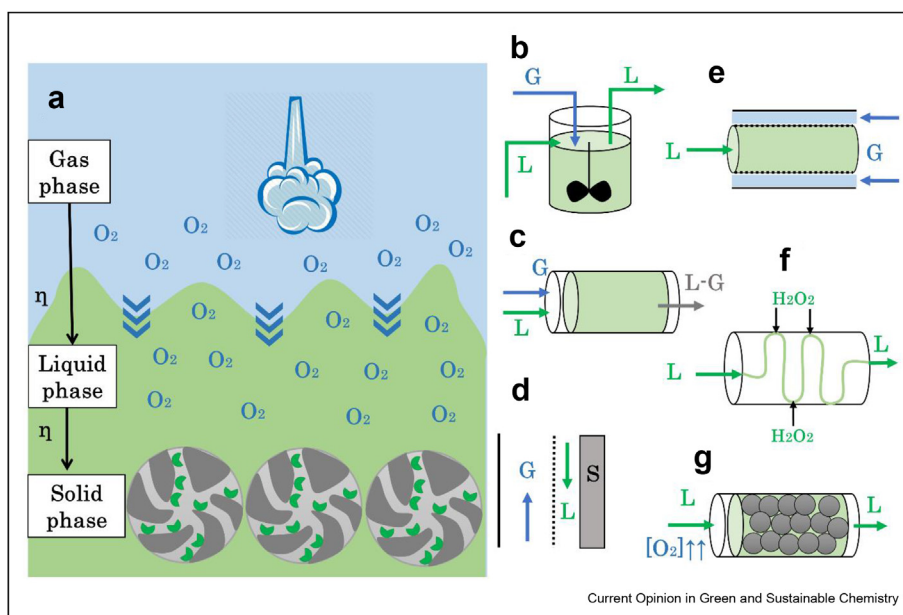


## Reactor engineering for intensified oxidative enzymatic reactions

Given the low oxygen solubility in the (aqueous) reaction medium, an external supply is needed, which affects not only at late phases of application but also at earlier stages of kinetic characterization [38–40,51]. Usually dispersive methods are used, where a gas phase (air, pure  $O_2$ ) is contacted with the liquid reaction phase and the transfer rate is given by the liquid-side mass transfer coefficient ( $k_L a$ ) [52–54]. The reaction rate is typically limited by low  $O_2$  transfer rate (OTR) and solubility in water [38,40,41], Figure 4a. For example, in industrial-scale stirred aerated reactors, a maximum value of  $5 \text{ min}^{-1}$  can be achieved (equivalently to a theoretical maximum of  $1.2 \text{ mM min}^{-1}$  when the liquid phase is fully deoxygenated and hence catalyst fully underused) [38,39,52,55]. Recently the dissolved oxygen concentration was corroborated as the rate-limiting factor of the oxidase-catalyzed oxidation of glucose studied in a stirred tank reactor (Figure 4b). Results showed that a threefold increase in the oxygen content of the feed gas improved the reaction rate by twice as much as a 10-fold increase in enzyme concentration [38]. However,  $k_L a$  can be enhanced via reactor design; OTR also depends on the driving force, scaling reciprocally to the available  $O_2$  and, hence, being entangled with the enzyme kinetics [38,40,41]. Improvement of the oxygen transfer capacity resides on reactor engineering [56–58]; Figure 4 schematizes some strategies. Literature offers disruptive advances

in reactor design in adjacent fields (chemical catalysis), in particular within continuous microreactors, that boosted  $k_L a$  [59–61], but biocatalyst integration compatible with reaction medium and matching characteristics of the reaction (catalyst dependent) with the OTR are usually a problem [62–65]. Other methods could be explored not only to increase the transport rate but also to ensure simultaneously sufficient concentration at the local level [60,66]. Using continuous-flow microreactors, the interfacial surface-to-volume ratio can be maximized, while the overall reaction time is minimized [57,65]. Using a segmented flow tubular reactor (Figure 4c) for enzymatic hydroxylation of trans-hex-2-enol, van Schie *et al.* [67] obtained a large turnover number ( $3 \times 10^5$ ) at low conversion. At a high conversion of 0.9, however, the space–time yield was low ( $0.25 \text{ mM min}^{-1}$ ) [67]. A continuous falling film microreactor can be applied for the oxidation of glucose catalyzed by free glucose oxidase [68], obtaining space–time yield of  $\sim 80 \text{ mM min}^{-1}$  at 20–30% conversion at a low turnover number ( $2 \times 10^3$ ), Figure 4d. Using the same type of reactor, a space–time yield of up to  $45 \text{ mM min}^{-1}$ , however, was achieved at a low turnover number of soluble glucose oxidase and low conversion [63]. An agitated cell reactor has also been applied to enhance the rate of biocatalytic oxidation reactions for the same transformation [51,69]. Another interesting reactor setup is the tube-in-tube configuration where the aqueous and gas phases are physically separated by a

Figure 4



**Reactor engineering for the intensification of oxygen-dependent oxidations.** (a) shows the multiphase features of the reaction. Limitation of the reaction rate can lie in the gas–liquid transfer or in the transfer rate (an oxygen gradient) into the solid phase. Different reaction engineering alternatives work on enhancing oxygen transfer rate or/and enhancing local supply into the solid phase. (b–g) show different reactor alternatives used and mentioned in text.

membrane [41,54,56,62,65], Figure 4e. The biocatalytic oxidation of glucose to produce sodium gluconate was recently applied in a rotating packed bed. The results indicated that the experimental value of  $k_{LA}$  was in the range of  $0.00984\sim 0.02758\text{ s}^{-1}$ , allowing a production rate up to  $6\sim 12\text{ g L}^{-1}\text{ h}^{-1}$  in the rotating packed bed, which was 7 times that in a stirred tank reactor [70].

Another current approach relies on the direct generation of oxygen. Oxygen can be produced in the reaction media itself for a bubble-free supply of gas based on the controlled decomposition of hydrogen peroxide catalyzed by catalase. Under the confinement of a porous particle or a flow reactor [71,72], this feature was exploited to boost the concentration of aqueous  $\text{O}_2$  beyond the limits practically achievable by gas–liquid transfer under safe and practical conditions [71–73]. The issue lies in the enzyme instability caused by a significant level of  $\text{H}_2\text{O}_2$  and keeping the balance between enzyme activity/stability and  $\text{O}_2$  gas formation. Multi-injection microchannel reactors can be a solution, Figure 4f, [73]. Another compatible option is the enhancement of the oxygen solubility, for example, by selecting the adequate solvent or cosolvent or by increasing the system pressure [38,74–76]. The use of a liquid-phase pressurized reactor allows reaching, at the same time, a high enzyme turnover ( $10^4$  mol product/mole enzyme), a high space–time yield ( $\geq 100\text{ g L}^{-1}\cdot\text{hr}^{-1}$ ;  $12\text{ mM min}^{-1}$ ), and a high conversion (0.8–1.0), Figure 4g. This is uniquely possible because the reactor does not involve trade-off between the  $[\text{O}_2]$  at steady state, the gas–liquid transport rate, and the residence time. Besides the enhanced supply of dissolved  $\text{O}_2$ , the pressurized reactor also involves kinetic intensification due to the increased  $[\text{O}_2]$  [77].

There are many examples of the intensification of  $\text{O}_2$ -dependent enzymatic reactions with free enzymes, as just commented, but there are very few examples of the application with immobilized enzymes. The limitation can be focused on the transport from gas to the liquid phase or on the transport to the solid catalytic phase and resulting intraparticle oxygen concentration, Figure 4a. The two main limitation steps of oxygen-dependent reactions can be studied comprehensively in microfluidic reactors, showing that the application of immobilized enzymes in microchannel wall-coated reactors not only boosts the volumetric activity but enhances the transport rate [78,79]. In a study with a wall-immobilized D-amino acid oxidase, it was shown by reaction diffusion time-scale analysis that the heterogeneously catalyzed reaction was always slower than diffusion of  $\text{O}_2$  to the solid surface, although at high volumetric activity of  $10\text{ mM min}^{-1}$  [78]. The application of microreactors in the oxygen-dependent reactions also increases the transport rate from the gas to liquid phase. This was demonstrated with a wall-immobilized falling film

microreactor. Advanced noninvasive optical sensing was applied to measure liquid phase oxygen concentrations in both in- and out-flow as well as directly in the microchannels to show how the reactor can supply up to  $100\text{ mM min}^{-1}$  of oxygen to the liquid phase [63].

Main challenge still resides in the compatible design of solid-supported enzymes and intensified reactors. This aim can be achieved by the conjoined design discussed in microreactors or by the integrated holistic development shown in Figure 1, where an active and stable heterogeneous catalyst is compatible/synergistic with the reaction setup. Very recent examples support this idea. One option is the enhancement of the oxygen solubility in pressurized liquid phase homogeneous reactors. In this way, biotransformations are conducted within a single liquid phase at boosted concentrations of the dissolved  $\text{O}_2$  (up to 43 mM). A packed bed reactor containing oxidase and catalase coimmobilized on porous beads was implemented to demonstrate catalyst recyclability and operational stability during continuous high-pressure conversion. Product concentrations of up to 80 mM were obtained at low residence times (1–4 min) [77]. In another example, an integrated study has been performed in stirred tank reactor configuration to study the P450 BM3-catalyzed hydroxylation of dodecanoic acid. Coimmobilized enzymes were applied in  $\text{O}_2$ -gassed and pH-controlled conversions at high final substrate concentrations ( $\geq 40\text{ mM}$ ). Running the reaction at  $\text{O}_2$ -limited conditions at up to 500-ml scale, a substrate feeding strategy based on  $\text{O}_2$  feedback control was applied. In that manner, high reaction rates of  $1.86\text{ g}\cdot\text{L}^{-1}\text{ h}^{-1}$  and near complete conversion ( $\geq 90\%$ ) of 80 mM C12:0 with good selectivity were achieved [50]. Enzyme-immobilized in the format of packed bed module reactors with in situ production of oxygen by controlled feeding of hydrogen peroxide has been recently shown [80]. In this work, galactose oxidase was used in conjunction with catalase to produce primary and secondary amines from various reactive intermediate carbonyls with high conversion ( $>95\%$ ). Furthermore, modular packed bed reactors including an oxidation unit have recently been implemented [81]. The space–time yield achieved in this work was  $2.59\text{ g L}^{-1}\text{ h}^{-1}$  with 15 min residence with an oxygen transfer rate of  $0.106\text{ }\mu\text{mol mL}^{-1}\cdot\text{min}^{-1}$ .

## Conclusion

Enzymatic biooxidation hosts a privileged situation in modern green chemistry. The exploitation of their benefits in sustainable chemical technology must be aligned with optimal catalyst design, reaction engineering, and process intensification. In  $\text{O}_2$ -dependent reactions catalyzed by immobilized enzymes, intensification is achieved primarily by enhancement of the solid catalyst productivity, the space–time yield in the reactor, and the process stability. However, multiple scientific and technological disciplines are available, and research and

development from early stages could be benefitted from a more holistic and integrated approach. For example, protein engineering provides access to enzymes with great properties, enzyme immobilization allows reaching stabilization under operational conditions, applied chemical kinetics assisted by advanced catalyst characterization allows identifying rate limiting step, material engineering and science of enzyme immobilization can boost catalyst productivity overcoming both enzyme inactivation and diffusional restrictions. Reactor engineering allows boosting OTR-limited space-time yield. Nevertheless, systematic studies integrating diverse dynamic phenomena and underlying the metrics useful for development across scales are still scarce. Metrics evaluated at an early stage of research and development are critical for anticipation. Ultimately, industrial implementation within the context of efficient sustainable processes (e.g. bulk chemistry) will require evaluation of sustainability metrics, also a different level of development, and cost-effectiveness analysis [82]. We also identified the need of comprehensive studies where new enabling technologies involving raw material pretreatment and downstream operation units are involved and integrated with the reaction unit because substrate conditioning, especially in the case of biomass, and product purification might be the cost-limiting step [82]. The body of evidence collected in this review is inspirational to build a development chain (Figure 1) that can bring the enzymatic biooxidation to a prominent level in the future green chemical manufacturing.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- of outstanding interest

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